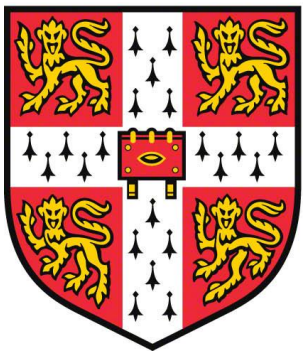
Investigating the Role of Human Cytomegalovirus Protein LUNA in Regulating Viral Gene Expression during Latency



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Abstract

Investigating the Role of Human Cytomegalovirus Protein LUNA in Regulating Viral Gene Expression during Latency

Human cytomegalovirus (HCMV) is a widespread human herpesvirus pathogen and prototypical member of the β-herpesvirus subfamily. Like all herpesviruses, the virus establishes a lifelong latent infection following host exposure, which has the potential to reactivate periodically and contribute to recurrent disease processes. In individuals with weak or compromised immune systems, such reactivation can lead to profound pathology. Understanding how latent infections are maintained is important for uncovering how HCMV causes disease. The study of viral genes that are expressed during latent infection grants insight into how latency is regulated and how it could be therapeutically targeted. To that end, this project has sought to evaluate the functional significance of one such viral gene termed LUNA in the context of latency. In models of experimental latent infection based on primary myeloid cells, levels of viral gene transcription were found to be significantly reduced following infection with LUNA deletion mutant viruses, consistent with corresponding observable changes in post-translational histone modifications over the viral promoters of latency-associated genes. Additionally, using luciferase reporter systems, latency-associated viral gene promoters became activated in response to the expression of wild-type LUNA. Together, these findings argue for a role of LUNA in regulating viral gene expression during latent HCMV infection. One possible mechanism by which LUNA may fulfil its role is by targeting cellular ND10 structures, known intrinsic inhibitors of herpesvirus gene expression, for disruption. In support of this, latently infected cells were found to be devoid of ND10, a phenotype that was recapitulated by the direct expression of wild-type LUNA. Furthermore, mutation studies confirmed the identification of a novel deSUMOylase activity encoded by LUNA that was responsible for mediating ND10 disruption. Use of a catalytically inactive LUNA mutant in transcriptional analyses of latent infection also generated similar results as with the LUNA deletion viruses. Overall, these data support the hypothesis that LUNA serves as an important regulator of viral gene expression during latency, which is likely linked to its ability to target ND10 structures for disruption, thus raising the possibility that inhibition of deSUMOylation may serve as a novel therapeutic strategy to target latent HCMV infection.

Jonathan C H Lau

February 2018

Declaration

I hereby declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as specified in the text.

The dissertation does not exceed the prescribed word limit of 60,000 words, excluding figures, photographs, tables, appendices and bibliography for the Degree Committee (Clinical Medicine and Clinical Veterinary Medicine).

Jonathan C H Lau
February 2018

This dissertation is dedicated to my grandparents and
the friendship and memory of Melanie White (1991 – 2011)

Acknowledgements

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Finally, and of no less importance, I would like to formally recognise the financial assistance kindly bestowed by the UK Medical Research Council, Teresa Rosenbaum Golden Charitable (Rosetrees) Trust and The Warden and Fellows of Robinson College in the University of Cambridge.

Jonathan C H Lau
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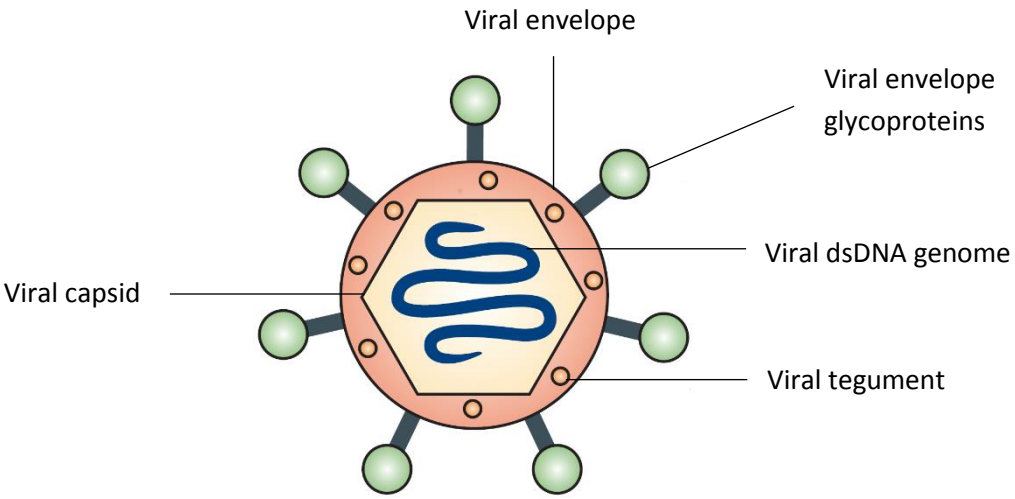
1. Introduction

1.1. HERPESVIRUSES

Evidence for the earliest known forms of herpesvirus infection can be traced as far back as the times of the Ancient Greeks, who coined the namesake "herpes" – from ἕρπειν (hérpein, "to creep") – in likely reference to the spreading nature of herpetic skin lesions[1]. Yet, it would not be until 1893, when French scientist Emile Vidal specifically recognised the person-to-person transmission of herpesviruses (in his case, herpes simplex) that an entire field of virology, with its own broad ramifications, would come to be foreshadowed. Today, the herpesviruses comprise an extensive group of large DNA viruses that are ubiquitous in nature and responsible for causing a myriad of diseases, some far removed from the mucocutaneous conditions they once originally stood for.

As illustrated in Figure 1.1, all herpesviruses share a distinctive virion architecture, consisting of a linear double-stranded DNA core surrounded by an icosahedral capsid, a tegument and a glycoprotein-containing envelope. In spite of this characteristic morphology, only recently has sequence data for herpesvirus genomes been instrumental in establishing a thorough taxonomy that now places three separate families, representing three major viral lineages, under the single principal order of *Herpesvirales*: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*[2]. These subdivisions closely mirror the phylogenetic branching order of their natural hosts; and thus, the *Herpesviridae* family contain viruses of higher vertebrates (namely mammals, birds or reptiles) while the *Alloherpesviridae* and *Malacoherpesviridae* families list viruses of amphibians and fish, or of bivalves, respectively. Notably, herpesviruses exhibit a remarkable degree of specificity, and it is typical for most to be restricted to a single host species. With more than 200 distinct herpesviruses identified to date – likely representing only a fraction of the total number that actually exists – the sheer diversity of their combined host repertoire is indicative of a long history of coevolution that has been estimated to span many millennia[3].

Figure 1.1 – Prototypical structure of herpesviruses



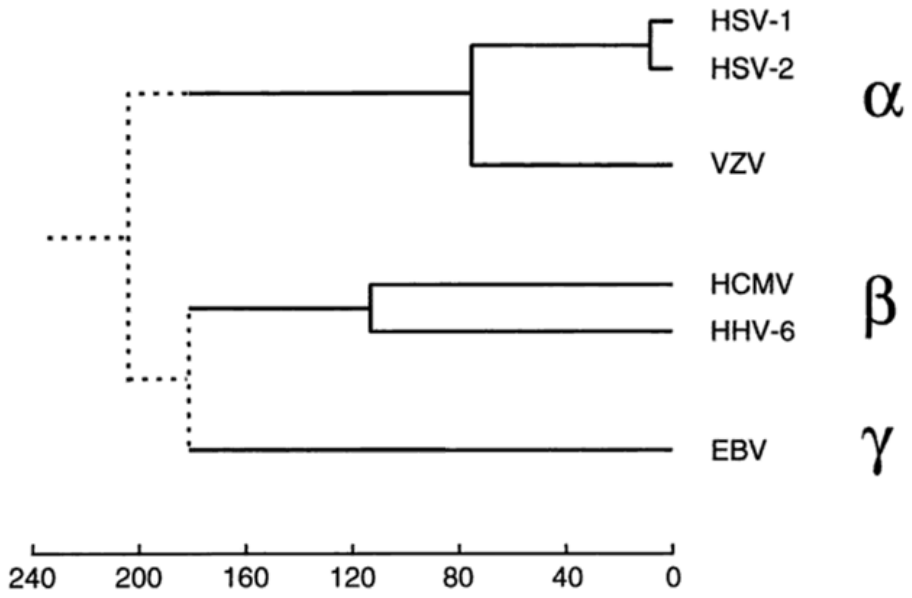
The general structure of the herpes virion consists of a linear double-stranded DNA core; an icosahedral capsid containing 162 capsomeres, including four protein subunits; the tegument, an amorphorus layer of phosphoproteins that is released into the host cell following infection; a lipid bilayer membrane envelope, which is derived from the *trans*-Golgi network of the host cell; and multiple glycoprotein complexes (at least 11) that are embedded in the lipid bilayer and which act as viral receptors.

The most intensely studied herpesviruses are members of the *Herpesviridae* family. This family is clustered into three major phylogenetic clades, which are taxonomically referred to as the *Alphaherpesvirinae* (α), *Betaherpesvirinae* (β) and *Gammaherpesvirinae* (γ) subfamilies. Each subfamily is divided into two or more genera; for instance, the *Alphaherpesvirinae* comprise the genera *Simplexvirus* and *Varicellovirus*. Among these subgroups are nine herpesviruses that have been identified in humans, constituting the so-called 'human herpesviruses' (Table 1.1). Extrapolation of evolutionary rates indicate that the α-herpesviruses separated from their parent lineage about 210 million years ago, before the lineage itself diverged approximately 30 million years later, giving rise to both the β- and γ-herpesviruses (Figure 1.2)[4]. Consistent with extensive evolutionary divergence, the human herpesviruses display a diverse range of biological properties, differing with respect to host cell range, length of replication cycle, and cytopathology. Though, one striking common feature is their ability to establish a latent infection and thereby persist for the lifetime of the infected host. While the mechanisms by which this is achieved vary across individual herpesvirus species, latency is nonetheless recognised as a principle hallmark of all herpesvirus infections.

Table 1.1 - Nomenclature of the human herpesviruses

Taxonomic designation	Common name	Viral subfamily
Human herpesvirus (HHV)-1	Herpes simplex virus 1	*α-herpesvirus*
HHV-2	Herpes simplex virus 2	*α-herpesvirus*
HHV-3	Varicella-zoster virus	*α-herpesvirus*
HHV-4	Epstein-Barr virus	*α-herpesvirus*
HHV-5	Human cytomegalovirus	*β-herpesvirus*
HHV-6 variant A or B	HHV-6 variant A or B	*β-herpesvirus*
HHV-7	HHV-7	*β-herpesvirus*
HHV-8	Kaposi's Sarcoma-associated herpesvirus	*γ-herpesvirus*

Figure 1.2 - Evolutionary timescale for the human herpesviruses



A phylogenetic tree for selected human herpesviruses deduced by McGeoch *et al.* (1995) using amino acid sequences from several well-conserved genes. Subfamily groupings are indicated by Greek letters, with Herpes Simplex Virus (HSV)-1, HSV-2, and varicella zoster virus (VZV) representing the α-herpesviruses, HCMV and human herpesvirus 6 representing the β-herpesviruses, and Epstein-Barr virus (EBV) representing the γ-herpesviruses. The proposed timescale is shown in millions of years before present (M-years BP). The oldest part of the tree is shown as a broken line to indicate lower confidence in data for this region. Adapted from McGeoch *et al*. (1995)

1.2. HCMV: BASIC VIROLOGY

Human cytomegalovirus (HCMV; also known as HHV-5) is one of the nine members of herpesvirus that infects humans. Along with its closest relatives, the roseoloviruses (HHV-6A, HHV-6B and HHV-7), HCMV belongs to the subfamily *Betaherpesvirinae*. The virus remains one of the best characterised members of this group and is often considered the prototypical β-herpesvirus, owing to its high host species specificity, distinctive cytopathology (cytomegaly), slow growth kinetics in culture, and *in vivo* tropism for haematopoietic tissue and salivary glands[5,6]. Since its isolation in 1956, the clinical presentation of HCMV disease has been widely catalogued and extended to include severe forms affecting both congenital and adult populations, wherein a limited control of viral replication, often linked to the immune status of the infected individual, constitutes the basis for pathogenesis[7].

1.2.1. Genome Structure, Organisation and Replication

At the genomic level, HCMV is the largest of any human viral pathogen, possessing a linear double-stranded DNA molecule at approximately 235 kbp in size[8]. The organisation of the genome is complex and comprises two regions of unique sequence, termed unique long (U_L) and unique short (U_S), which are separated by internal repeat sequences (IR_L and IR_S) and bounded by terminal repeat sequences (TR_L and TR_S) to yield the overall configuration: 5'– $TR_L – U_L – IR_L – IR_S – U_S – TR_S$ –3'. Located between the UL57 and UL69 genes of the U_L region is a *cis*-acting site that serves as the only known origin of lytic DNA replication (ori_{Lyt}), which also happens to be one of the largest among the *Betaherpesvirinae*[9,10]. Due to recombination events occurring naturally in replicating DNA, the inversion of the U_L and U_S regions with respect to each other permits the HCMV genome to exist as one of four possible isomers; however, while this is dispensable for viral replication, the importance of viral genome isomerisation remains largely unknown.

Determination of the genetic content of HCMV has been a protracted process, serving as a constant reminder of the unbridled complexity that surrounds the coding potential of the virus. Sequence annotation of the clinical (hitherto referred to as low-passage) strain Merlin, which has become the reference strain for wild-type HCMV, lists approximately 170 protein-coding open reading frames (ORFs) in addition to 39 herpesvirus 'core' genes[11]. The core genes,

which are orthologous across all members of the human herpesvirus family, chiefly encode proteins involved in viral DNA replication as well as structural components of the virion. The vast majority of remaining HCMV genes however, while dispensable for growth *in vitro*, are replete with important accessory functions, notably involved in evading host immune responses, mediating virus cell tropism, and regulating latency. These comprise several sets of genes – grouped into 15 gene families – that unsurprisingly share homology with other cellular genes and show conservation among other primate cytomegaloviruses (Figure 1.3). Nevertheless, despite this requisite genomic data, HCMV is increasingly being recognised as having a multifaceted transcriptome[12]. To this end, ribosome profiling has led to the suggestion that up to 604 additional protein-coding ORFs may exist, most of which are very short and located upstream of previously identified ORFs[13]. Moreover, the transcriptome has been linked to noncanonical translation events, such as the production of polyadenylated non-coding RNAs, overlapping anti-sense transcripts, and also a variety of non-polyadenylated RNAs, such as microRNAs, many of whose roles are yet to be fully characterised, but appear to be regulatory[14,15]. Indeed, it is worth noting that efforts to establish an accepted reference strain were historically marred by the progressive realisation that diverse sequence variation occurs throughout HCMV genomes, restricting their global usage. Of particular note is that high-passaged (i.e. laboratory-derived) strains of HCMV – which were among the first to be sequenced – had been found to acquire significant mutations, deletions and rearrangements as a result of extensive adaptation to laboratory cell culture compared to low-passaged clinical isolates[16]. Naturally, the latter are used conventionally for research in an attempt to preserve the characteristics of wild type HCMV. Yet, the propensity for rapid mutation in HCMV is striking with instances of mutations being reported in even low-passaged strains, such as TB40/E exhibiting frame shift mutations in UL141 relative to the reference strain Merlin[17]. Nevertheless, several studies have since been able to demonstrate the presence of discrete clusters of polymorphism within a subset of HCMV genes, which has accordingly allowed multiple strains of HCMV to be classed as distinct genotypes (Table 1.2)[18–20].

Figure 1.3 - Open reading frame arrangement of HCMV low-passage isolates

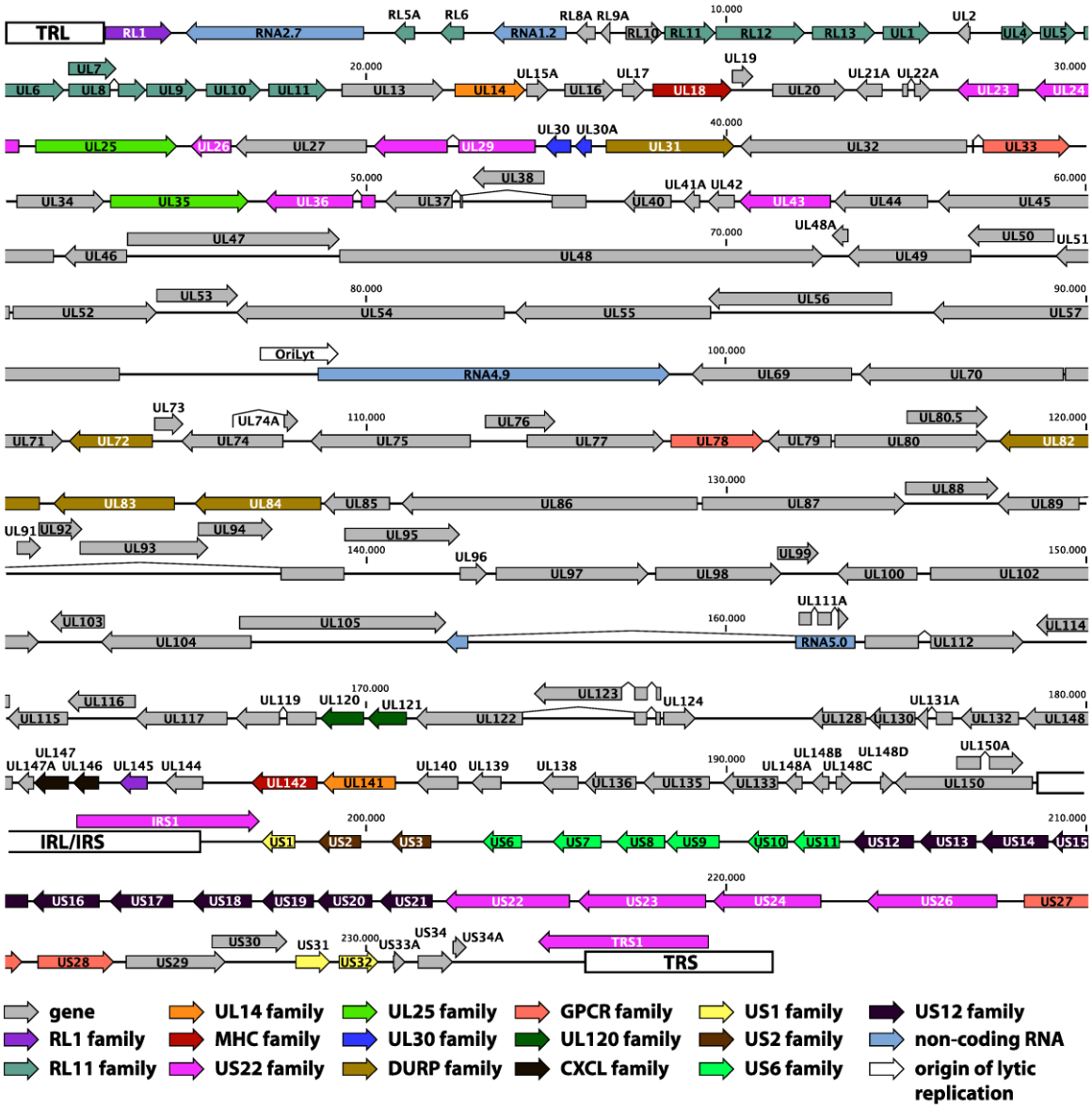


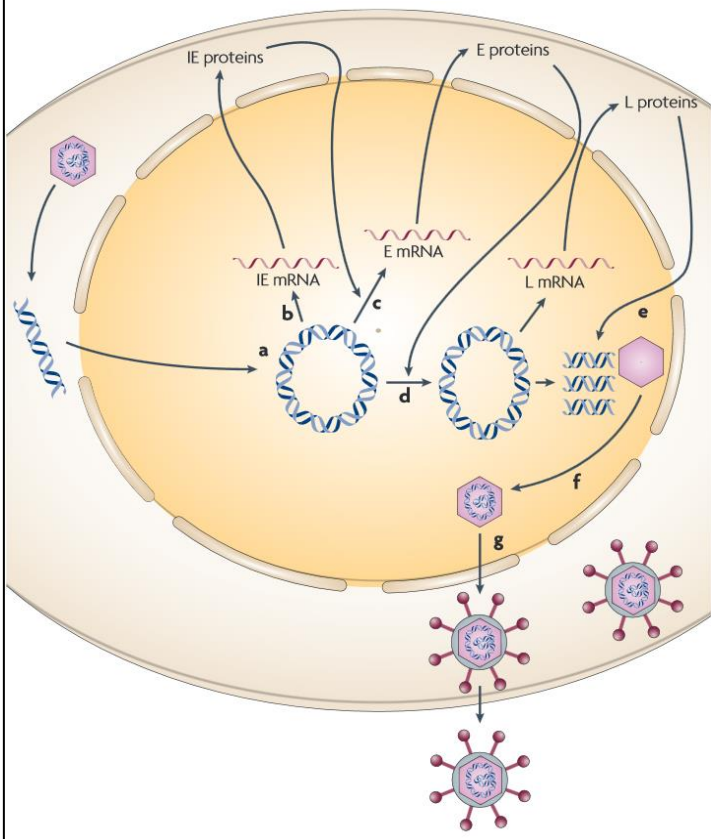
Figure 3. Genome annotation of the low-passage (clinical) HCMV strain Merlin (GenBank accession number: NC_006273). The genome is visualised as a single line, with nucleotide positions given in bps. Terminal (TRL,TRS) and internal (IRL/IRS) repeat regions are indicated by white boxes. Genes are represented by arrows, with up to 15 colours assigned to individual gene families. Also represented by the figure are four large non-coding RNAs and the origin of lytic replication (OriLyt). Reproduced from Sijmons *at al.* (2014) under an open access license (CC-BY).

Table 1.2 - Properties of selected HCMV strains and their bacterial artificial chromosome (BAC) clones [Adapted from: Frascoli *et al*. Methods Mol Biol (2014)]

	Strain	Tropism	Raw Sequence	BAC Clone	Genes altered	Ref
Laboratory Strains:	AD169varUC	Restricted	FJ527563.1	-	RL5A, RL13, UL131A, UL140-144	21
	AD169varUK	Restricted	X17403.1 BK000394	-	RL5A, RL13 (UL42-43), UL131A, UL133-150	21
	Towne	Restricted	FJ616285.1	$Towne_{BAC}$	UL133-146 BAC: IRS1; US1-12	22,23
Low passage (Clinical):	TB40/E	Extended	-	TB40-BAC4 / EF999921.1	RL6, RL13, (UL141) BAC: IRS1, US1-6	24
	VR1814	Extended	GU179289.1	FIX-BAC / AC146907.1	BAC: IRS1, US1-6	25
	Merlin	Restricted	AY446894.2 NC_006273.2	pAL1111	RL13, UL128	26,27

As with other herpesviruses, the life cycle of HCMV exhibits both latent and lytic / productive phases, each denoted by distinct patterns of viral gene expression[28]. During latency, viral gene expression is tightly controlled, with few but well-defined latency-associated transcripts (LATs) being expressed that do not coincide with production of infectious virions. By contrast, gene expression during lytic replication is extensive and unrestricted, following a temporally regulated cascade that culminates in the lysis of infected cells and the release of mature viral progeny. The products of these genes are assigned to one of three classes based upon their expression kinetics: immediately-early (IE), early (E) or late (L) (Figure 1.4). In brief, IE genes – which arise independently of *de novo* viral protein synthesis and are thus the first to be expressed – encode regulatory transactivating factors that are necessary to drive the viral-cell fate decision towards lytic replication[29]. The proceeding set of E genes, whose activation is dependent on prior IE gene expression, contribute towards essential processes such as viral DNA replication and repair as well as immune evasion. Encoded among these is a conserved set of six core replication proteins, namely: the viral DNA polymerase (UL54), the polymerase-associated processivity factor (UL44), the single-stranded DNA (ssDNA) binding protein (UL57), and the heterotrimeric helicase-primase complex formed by HP1 (primase; UL105), HP2 (primase-associated factor; UL70) and HP3 (helicase; UL102)[30]. Finally, L genes, which are expressed following viral genome synthesis, are implicated in the assembly, morphogenesis and egress of progeny virions.

Figure 1.4 - Overview of HCMV lytic infection cycle



a. Parental viral DNA enters the host cell nucleus and rapidly assumes a circular, episomal configuration. **b.** Viral immediate-early (IE) genes are the first to be expressed, the transcription of which arises in the absence of *de novo* virion production and involves a hijacking of host RNA polymerase II. **c.** IE proteins are transported into the nucleus and drive the next phase of early (E) gene expression, whose products include proteins required for viral DNA replication. **d.** DNA replication stimulates the expression of the late (L) genes, the majority of which encode viral structural proteins. **e. + f.** Virion capsid assembly and encapsidation of viral progeny take place in the nucleus. **g.** Virions egress from the nucleus and the cell, usually resulting in cell lysis. [Adapted from: Knipe *et al.* Nat Rev Microbiol (2008)]

The process by which HCMV replicates its genome may be described in reference to its lytic replication cycle. Firstly, in order to gain entry into cells, incoming virus particles undergo multistep binding at the cell surface that results in membrane fusion and penetration[31]. This liberates nucleocapsids into the cytosol, along with several tegument proteins. The nucleocapsids are then transported to the nuclear pore complex along the host microtubule network, where uncoating of the viral DNA takes place[32]. Once released into the nucleus, the otherwise linear DNA rapidly (within 2 hours post infection) becomes circularised and interacts with host cell histone proteins to form nucleosomes that partially resemble cellular chromatin[33,34]. The resulting viral episomes serve as templates for virus transcription and replication, which have been found to occur within discrete nuclear inclusions termed replication compartments[35].

Replication compartments develop adjacent to small subnuclear structures known as promyelocytic leukaemia bodies (PML-NBs) or nuclear domain 10 (ND10), and come to occupy large parts of the nuclear space at late times post infection[36,37]. Their formation and growth signify a dynamic reorganisation of the nuclear architecture that includes, for

instance, rearrangement of host cell nuclear proteins and partitioning of cellular chromatin, which, overall, help to concentrate factors and processes required for viral replication[35,38,39]. Notably, HCMV DNA synthesis has been found to occur at the periphery of replication compartments, with replicated DNA subsequently becoming localised to the interior of compartments[40]. At present, it is understood that HCMV genome replication is initiated at the ori_{Lyt}, and is dependent on a viral complex formed by UL84 and the key regulatory protein IE2[41,42]. In addition, four viral phosphoproteins encoded by UL112-113 (namely pp34, pp53, pp50 and pp84) become associated at pre-replicative sites – known structural precursors of replicative compartments – to coordinate assembly of the viral replisome[43]. This leads to the eventual recruitment of the aforementioned core replication proteins that participate in the synthesis of viral genomic DNA and which remain associated with the replication compartment throughout the entire infection[44]. Here, the helicase-primase complex unwinds the template DNA, thereby generating the primer from which the UL54-UL44 complex will synthesise the leading strand, while UL57 enforces strand separation to prevent any unnecessary reannealment[45]. During this period, other HCMV accessory proteins as well as host cellular factors may come to be involved, such as viral deoxyribonuclease UL98, and host SWI/SNF family of chromatin remodelers, which in particular, are crucial for granting replication machinery access to compacted DNA[46,47]. Thus, as viral DNA synthesis proceeds, long head-to-tail concatemers containing the four genomic sequence isomers are produced, serving either as new templates for ongoing cycles of replication or targets of cleavage for viral assembly. In the case of the latter, a viral terminase complex composed of viral UL89, UL56 and UL51 is responsible for processing concatemeric DNA into unit-length molecules by cleaving at DNA packaging signals called *pac1* and *pac2*[48,49]. These *cis*-acting sites are conserved among herpesvirus genomes and are crucial for viral DNA maturation[50,51]. While binding of UL56 to *pac1* and *pac2* appears to be important for initiating viral genome packaging, the mechanisms underlying subsequent encapsidation of nascent DNA into preformed capsids within the nucleus are not fully known[48,52]. Yet, it is clear that progeny HCMV genomes are stripped naked before being packaged, as mature linear DNA is devoid of any nucleosomes within the completed virus particle[53]. After nucleocapsids have been translocated from the nucleus, HCMV virions undergo maturation in the cytosol. In this regard, the essential processes of viral nuclear egress and morphogenesis remain poorly understood. One generally accepted view is that HCMV participates in two distinct envelopment processes; the first of which is transient to allow viral nucleocapsids to exit the nucleus, with the second being indefinite as virions bud into a cytoplasmic assembly compartment that primes them for eventual release from the cell[54,55].

1.2.2. Clinical pathogenesis of HCMV disease

HCMV is epidemiologically prevalent, infecting approximately 60–100% of the global population depending on specific demographics, such as age, gender and socio-economic status[6]. Although the vast majority of HCMV infections are asymptomatic, populations most at-risk of pathology include the developing foetus, HIV-infected hosts, and recipients of bone marrow and solid-organ transplantation[56]. Together, these groups represent a significant source of morbidity and mortality worldwide for which HCMV serves as an important viral pathogen.

In the clinic, HCMV may directly affect a diverse range of anatomical sites, with the lung, gastrointestinal tract, central nervous system and retina, among others, being linked to overt end-organ disease[57]. Here, the underlying pathology is widely believed to be driven by uncontrolled lytic viral replication that culminates in a clear, cytopathic effect[58]. In support of this, histological analysis of affected organs obtained from infected individuals typically reveals the presence of characteristic viral inclusions and necrotic damage. Moreover, the administration of antiviral therapy (e.g. ganciclovir) to patients with active viral replication and disease leads to a decrease in viral load and an improvement in symptomatology, which contrasts with use of steroids that can promote disease[59,60]. Interestingly, the frequency of particular disease presentations vary between different cohorts of patients with, for example, HCMV retinitis being more prevalent in the HIV/AIDS population, HCMV pneumonia manifesting after haematopoietic stem cell transplantation, and HCMV neurological sequelae, including sensorineural hearing loss, commonly exhibited by infected infants. However, HCMV infection has also come to be associated, albeit statistically, with other so-called indirect effects, such as cardiovascular disease and morbidities related to normal aging[61,62]. Although the possibility of attributing specific organ dysfunction to HCMV infection cannot be discounted in such cases, many studies have nonetheless sought to investigate the role of HCMV as a co-factor in their pathogenesis and less so an epiphenomenon. Notably, the potential contribution of HCMV towards immunopathology appears to be a common factor that links a number these particular disease associations[63]. Throughout its course as a lifelong persistent infection, for instance, it has been speculated that chronic immune surveillance for HCMV may generate an increased abundance of activated T cells that can mediate inflammatory attacks on bystander cells, such as the arteriolar endothelium[64,65]. Additionally, in elderly patients, a reduced number of naïve T cells would make seropositive people less able to respond sufficiently to vaccines, resulting in excess mortality during periods of influenzal or pneumococcal infectious outbreaks[65].

As eluded to above, the immune status of the infected individual is a major determinant for the clinical expression of HCMV-associated disease, such that those with compromised immune systems are left especially vulnerable[57]. Early clinical data obtained from patients undergoing allografts showed that decreases in adaptive immune responses – brought about by the necessary administration of immunosuppressive drugs – were strongly linked to the development of clinically significant HCMV infection and invasive end-organ disease[66,67]. In this respect, the passive transfer of antiviral antibodies or *in vitro* expanded HCMV specific $CD8^+$ T-cells was found to confer a protective effect in infected allograft recipients[68,69]. Similarly, HCMV is regarded as a common opportunistic infection in individuals living with HIV, particularly as $CD4^+$ T cell counts fall below 100 cells/mm^3 [70,71]. Given that antiretroviral therapy, which helps to maintain the CD4 count above 100, is regarded as an effective prophylactic strategy against HCMV disease, this further demonstrates how adaptive immune surveillance is important in controlling the virus. For the foetus and newborn infant, it has been suggested that the immaturity of the adaptive immune system present during that developmental period may explain their susceptibility to HCMV infection[72]. In support of this, the magnitude of the $CD4^+$ T cell response in the foetus has been correlated against the severity of HCMV disease[73,74]. Additionally, during congenital HCMV infection, the number of HCMV specific $CD4^+$ T cells are very low or undetectable in infants, and furthermore, are unable to effectively produce inflammatory cytokines, such as IFN-γ and IL-2[75]. Consequently, these factors are believed to negatively affect viral clearance, thereby increasing the risk of HCMV disease progression.

Advances in next-generation sequencing (NGS) technologies have enabled the comprehensive study of genetic diversity across the entire HCMV genome and ongoing work has examined whether this may serve as an important HCMV virulence factor[76]. Longitudinal changes in the global HCMV population, for example, are known to contribute to host immune evasion. Differential properties conferred through polymorphisms in viral glycoproteins, most of which are hypervariable, may give rise to various epitopes, each possessing altering specificities for neutralisation from existing humoral responses[77–82]. Yet, further to this, recent studies have revealed that HCMV exhibits significant levels of genetic diversity within a single individual (i.e. at the intrahost level), demonstrating that HCMV evolution manifests on a short time scale, in the order of days or months[83,84]. Indeed, it is clear that mixed HCMV infections, which feature multiple genotypes within individual hosts, is common phenomenon affecting up to one half of all HCMV infections in a wide range of human populations, such as infants with congenital disease, people with HIV/AIDS, transplant recipients, children and

adults[76]. A number of plausible mechanisms underlying this mode of evolution haven suggested, each of which demonstrate clear potential to promote HCMV spatiotemporal evolution, and include: low frequency generation of *de* novo mutations, reinfection, genetic recombination and natural selection. Although no clear conclusion on the possible impact of any particular single genotype on disease has yet been reached, it is becoming increasingly understood that mixed genotype infections do in fact correlate with increased viral loads, HCMV disease severity and even progression to AIDS in people with HIV infection[85–87].

For most healthy individuals, primary infection with HCMV generally manifests as a period of productive infection that is quickly limited by the host immune response, leaving behind a pool of latently infected cells that contribute towards lifelong persistence. Since it is not uncommon to observe the involvement of a range of organ systems in cases of overt pathology, this suggests that the virus is able to undergo efficient dissemination *in vivo*[65]. Indeed, the extent to which viral host spread can be attributed to cell-associated transmission between infected cells and uninfected tissues is supported by findings that very little extracellular virus is detectable in the plasma of HCMV-infected individuals, and furthermore, depletion of the peripheral blood compartment prevents lateral transmission of the virus[88–90]. Models describing HCMV pathogenesis during primary infection, but also reinfection, are therefore consistent in highlighting the importance of several host cell types that are likely to be involved in mediating the systemic spread of the virus[91]. For instance, epithelial cells lining the nasopharynx and urogenital tract, along with endothelial cells of the vascular tree, are regarded as major sites of HCMV acquisition, whose excretions also have the potential to transmit virus onwards through known oral, sexual and blood-borne routes[92,93]. By contrast, circulating blood leukocytes are believed to play an essential role in seeding the virus haematogenously, bringing into reach of infection the parenchyma of various organs such as the brain, retina, lung, liver and gastrointestinal tract, which have all been shown to manifest HCMV-associated disease[94]. Indeed, an HCMV cell-associated viraemia occurs early upon infection, with viral DNA being found predominantly in peripheral blood monocytes and polymorphonuclear leukocytes (PMNs) but not lymphocytes[95–97]. Though neither monocytes nor PMNs are able to support productive HCMV replication, they are still able to participate in the uptake and transport of infectious viral particles, which can then be transmitted focally through cell-associated contact[98–100]. Monocytes, in particular, have been characterised as highly motile carriers that feature as the most dominant infiltrating cell type seen in HCMV-infected tissues and are otherwise regarded as the principle cell type responsible for distributing the virus within the host[101]. Additionally, as a member of the myeloid lineage, both monocytes and their associated myeloid progenitors, are also critical in the establishment

of HCMV latency[102,103]. Thus, by acting as lifelong sources of periodic viral shedding, these particular infections underlie the pathological basis for causing recurrent disease.

1.3. VIRAL-HOST CELL TROPISM

HCMV possesses a broad cellular host range that is clearly notable from a clinical perspective. How the virus achieves such promiscuous cell tropism forms the basis of much ongoing study, which has contributed towards a better understanding of its basic pathogenicity.

To begin with, all enveloped viruses gain entry into host cells by releasing their nucleocapsids into the cytosol[104]. This requires direct fusion between the virion envelope and cellular membranes, which may either occur at the level of the plasma membrane or endocytotic vesicle. Glycoproteins found in the virion envelope are responsible for mediating the viral cell entry process by binding to cognate cellular entry receptors, thereby triggering a sequential activation cascade that results in membrane fusion. For all herpesviruses, the 'core' fusion machinery comprises the heterodimeric glycoprotein complex gH/gL and the class III fusogen gB. According to the working hypothesis, multiple gH/gL complexes may exist to regulate the fusogenic activity of gB[105,106]. Thus, in the case of HCMV, two alternative forms of the gH/gL complex have been described and are regarded as important determinants of virus host cell tropism[107,108]. The first of the two complexes, gH/gL/gO, is a heterotrimer formed by gH/gL and a heavily glycosylated protein (gO; encoded by *UL74*), while the second, gH/gL/UL128-131, is a pentamer formed between gH/gL and three small glycoproteins (encoded by *UL128*, *UL130*, and *UL131*)[109–111].

Much prior research on the roles of each HCMV gH/gL-containing complex have been crucial in establishing the concept of a functional dichotomy for HCMV cell tropism. On the one hand, viral entry into fibroblasts (the accepted standard cell type for HCMV tissue culture) depends on the presence of the gH/gL/gO trimer, which facilitates direct fusion at the plasma membrane[112]. On the other hand, entry into more clinically relevant cell types such as epithelial cells, endothelial cells and myeloid cells, requires the gH/gL/UL128-131 pentamer, which instead, promotes the internalisation of HCMV through endocytotic pathways, thereby necessitating direct fusion at the vesicular membrane[113–116]. This dichotomy is broadly challenged, however, by the observation that gO-null mutants show a massive loss of cell-free infectivity with respect to all of the above cell types[117]. Hence, while both complexes are likely to be involved in conferring – or broadening, in the case of gH/gL/UL128-131 – the

relevant host cell tropism through their own respective receptor binding interactions, it is now understood that only the trimer preserves the core gH/gL function (i.e. activating gB-mediated fusion), which is strictly required for entry into all target cell types[118]. Interestingly, cell-associated spread *in vitro* is not compromised when gH/gL/gO is lacking. Rather, the removal of gO from a virus background impairs the release of infectious virions from infected cells, which causes viral spread to become focal and instead dependent on the pentameric complex[117,119]. This is reminiscent of recent work performed *in vivo* using murine cytomegalovirus (MCMV), demonstrating that while gH/gL/gO is important for the initial infection of mice, it is dispensable for subsequent viral spread to distal tissues and organs[120]. Recently, the cellular receptor for gH/gL/gO was identified as platelet-derived growth factor receptor alpha (PDGFR-α), which is expressed on fibroblasts, but not epithelial cells[121]. Although the identity of an equivalent receptor for gH/gL/UL128-131 remains unknown, there is evidence supporting the role of the pentamer in activating specific receptor-mediated signalling pathways that are otherwise necessary for the internalisation of HCMV into myeloid cells[122,123].

Because the HCMV *UL128-131* locus (encoding the pentamer-specific glycoproteins) is known to be genetically unstable during viral passage in fibroblast tissue culture, it is not surprising that the dichotomy concerning HCMV host cell tropism also exists on a broad scale, outside that of its own mechanistic underpinnings[26]. As such, frequently passaged laboratory-adapted strains of HCMV differ significantly from unpassaged clinical isolates, with the former having a restricted host cell range (i.e. poorly infecting endothelial, epithelial and myeloid cells, but not fibroblasts), while the latter retaining the ability to competently infect a broad range of cell types; a phenomenon that has previously been regarded as a surrogate marker of viral pathogenicity or attenuation *in vivo*[124–127]. Accordingly, numerous inactivating mutations have been detected within the UL128, UL130 and UL131 ORFs of laboratory-adapted strains, such as Towne and AD169, which correlate with an inability to form a functional gH/gL/UL128-131 complex, whereas gH/gL/gO remains unaffected[21,22]. Moreover, the prototype low-passage strain Merlin has been found to sustain a truncation of *UL128* after only being passaged three times in fibroblasts, which is also associated with reduced incorporation of the pentamer in the virion and a loss of infectivity in epithelial cells[128]. It is therefore clear that any attempt to develop low-passage clinical isolates for the purposes of generating a laboratory strain of ‘wild type’ HCMV – which should otherwise be in possession of a naturally-occurring phenotype (let alone genetic sequence) – is significantly hampered by the rapid occurrence of genetic adaptation to cell culture[129]. Nevertheless, most

laboratory-adapted strains of the virus were historically developed by the process of serial passage in fibroblasts in an attempt to generate potential attenuated vaccine candidates. As such, the finding that sequential mutations associated with prolonged adaptation to fibroblasts is linked to greater resulting yields of cell-free virus, which can be used exogenously for *in vitro* experimentation, is crucial for supporting their continued application[130].

Moreover, it is now clear that both endothelial cell tropism and leukotropism can be reinstated in laboratory-adapted strains Towne and AD169 after they have undergone adaptation to growth in endothelial cell culture[131–133]. Hence, effective experimental design should necessarily take into account the choice of tissue type when wishing to generate experimental stocks of virus. Surprisingly, it has been reported that progeny virions produced by infected fibroblasts display a broader host cell tropism than those shed from infected endothelial cells, and that this relates to the cell type-dependent release of heterogeneous viral subpopulations that vary in terms of overall abundance of the pentamer[134]. While these findings are likely to have important clinical relevance with respect to understanding how instances of tropism switching may impact upon subsequent viral dissemination *in vivo*, they do not detract from the fact that repeated passage of broadly tropic HCMV strains can lead to loss of gH/gL/128-131 and a more stringent host cell range[135]. To this end, it is worthwhile restating that though clear interstrain differences in HCMV cellular tropism occur as an artefact of extensive cell culture, even heavily laboratory-adapted strains like AD169 are not rendered completely non-infectious and can still infect non-fibroblast cell types to a degree[136].

1.3.1. Lytic Replication and Control of the Major Immediate Early Promoter

During HCMV infection, the virus may undergo one of two distinct transcriptional programs, resulting in either latency or lytic replication. As previously stated, latency represents a generally immunologically silent mode of persistence, which is broadly asymptomatic. In contrast, lytic replication underscores the production of new viral progeny, which leads to lysis of the host cell. While both states of infection are critical in mediating viral pathogenesis and spread, it is abundantly clear that clinically associated disease stems from a productive state of infection[137]. Accordingly, since this process proceeds in a highly coordinated, albeit cascade-like manner, involving the sequential activation of three temporal classes of lytic viral genes (IE, E and L), it is important to consider the mechanisms and events that underlie its induction.

To begin with, levels of permissiveness for productive HCMV infection are highly dependent on the target cell type, as in particular, certain cells are known to maintain the virus in an otherwise latent state. However, it is well established that commitment to lytic replication is predicated on the ability to support IE gene expression, not least because IE gene products, most notably the nuclear phosphoproteins IE1-72 kDa (IE1; *UL123*) and IE2-86 KDa (IE2; *UL122*), are essential for mediating the subsequent expression of E and L lytic genes, which are required to complete productive replication[138].

Broadly, the HCMV IE1 and IE2 proteins function as major viral regulatory factors, which are critical not just in controlling lytic infection, but also in priming the cellular milieu for virus production[139]. In detail, the IE1 protein synergises with IE2 to promote the transcriptional activation of early viral genes, though it is largely dispensable for HCMV replication at high multiplicities of infection (MOI)[140,141]. Contrastingly, the IE2 protein functions as a master regulator of HCMV transcription, one that is absolutely essential for facilitating progression down the lytic gene cascade[23,142]. The transactivation mediated by IE1 and IE2 appears to involve discrete and overlapping interactions with the basal cellular transcriptional machinery (including host chromatin remodelers), which have been shown to correlate with increased levels of activity from early viral promoters[29,143–146]. Beyond this, both viral factors have been implicated in modulating the host cell cycle during HCMV infection[147]. Only terminally differentiated cells in the G_0/G_1 phase of the cell cycle are fully permissive for lytic replication, as cells infected during the latter G_2 or S phases fail to express IE genes, resulting in the development of abortive infections[148]. IE2, in particular, is believed to promote cell cycle progression from G_0/G_1 to G_1/S, whereupon it arrests the cell cycle at the G_1/S interface, thus allowing the virus to increase the vital pool of dideoxynucleoside triphosphates and biosynthetic enzymes required for DNA replication, while also subverting it (from the cell) for its own use[149]. Direct expression of IE2 has been shown to strongly activate an array of E2F-responsive genes that are principally involved in regulating DNA precursor synthesis and control of the cell cycle[150]. This activation is believed to be facilitated by the binding of IE2 to the tumour suppressor protein Rb of the retinoblastoma (RB) family of pocket proteins, which inactivates it, thereby relieving the repression of genes sensitive to E2F activity[151,152]. In turn, to promote cell cycle arrest at the G1/S checkpoint, HCMV engages in the stabilisation of another well-known tumour suppressor: p53[153]. Here, IE2 may target the p53-specific E3 ubiquitin ligase mdmd2 for degradation, which stops it from forming an auto-regulatory feedback loop with p53[154,155]. Additionally, IE1 and IE2 participate in blocking the induction of host genes involved in mounting host antiviral

immunity[156]. To that end, IE1 can antagonise the type I interferon (IFN) response by forming complexes with human signal transducer and activator of transcription (STAT) proteins, such as STAT2, that are involved in the Janus kinase (Jak)-STAT signalling pathway, and redirecting them to nuclear chromatin compartments[157,158]. Whereas IE2 can target intranuclear NF-κB and prevent it from mediating the expression of inflammatory cytokines, such as IL-6 and IFN-β, and genes responsive to TNF-α, all of whose promoters possess NF-κB binding sites[159–161].

Expression of IE1 and IE2, along with a vast majority of other IE gene transcripts, originates from a region located in the U_L portion of the viral genome, termed the major IE (MIE) locus. This locus gives rise to a common primary transcript, composed of five exons, that undergoes alternative splicing to generate a number of distinct mRNA species (Figure 1.4). Among these, IE1 and IE2 are its most abundantly expressed products; both sharing 85 N-terminal amino acids corresponding to MIE exons 2 and 3, but containing distinct C-termini encoded by exon 4 in IE1 or exon 5 in IE2. For reasons not fully understood, this pattern of IE gene splicing is conserved among cytomegalovirus species even in the noted absence of amino acid homology[162]. The expression of the MIE precursor RNA is controlled by an upstream regulatory element located within the MIE locus, known as the MIE promoter/enhancer (MIEP). The MIEP is a highly potent and complex domain that is able to function in a tissue- and cell-type specific manner[163]. Hence, it contains binding sites for a diverse range of signal-regulated stimulatory and inhibitory eukaryotic transcription factors, whereby the cell-type specific promoter/enhancer activity is linked to the availability of appropriate factors in a given cell type (Figure 1.5)[164].

Figure 1.4 - Overview of transcripts originating from the MIE gene locus

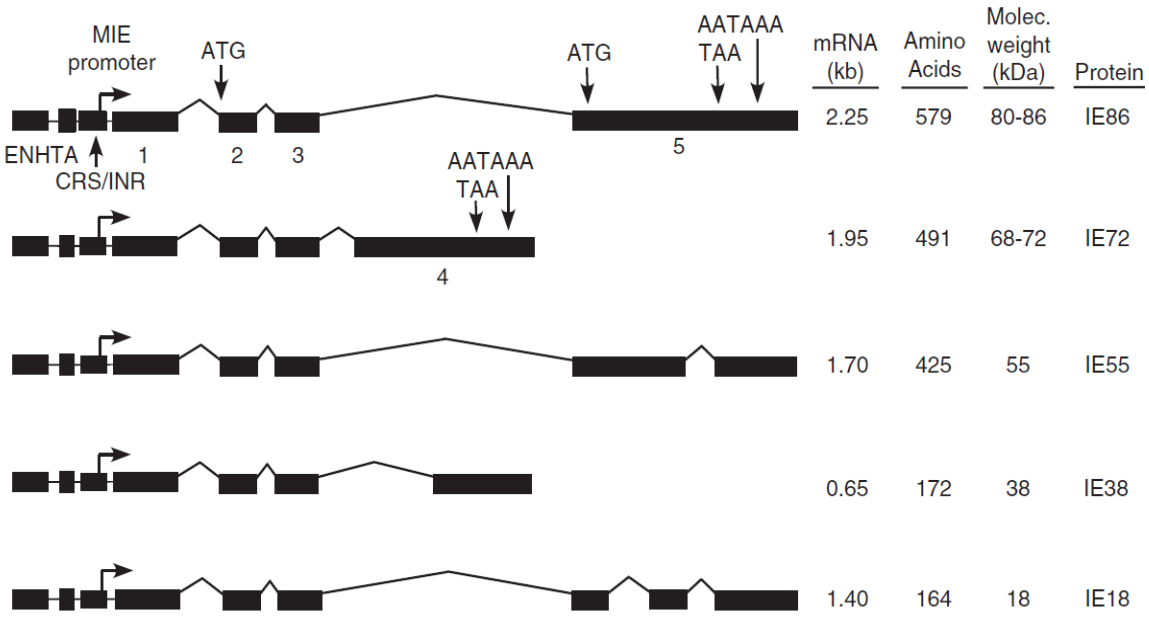


Figure 4. The MIE gene locus generates a single primary transcript that undergoes differential splicing and polyadenylation to produce multiple mRNA species. The IE1 and IE2 mRNAs, which respectively code for IE72 and IE86, are the most abundantly expressed transcripts. They share the first three exons, with IE1 containing exon 4 and IE2 containing exon 5. Minor isoforms are also produced from the IE1 and IE2 genes as illustrated; however, less is known about their functions due their relative low abundance, which makes them difficult to study independently. ENH: enhancer; TA: TATA box; CRS: *cis* repression sequence; INR: initiator; MIE: major immediate early; ATG: start codon; TAA: stop codon; AATAAA: polyadenylation signal; kb: kilobases; kDa: kilodaltons. Reproduced from Stinski and Petrik (2008) with permission from Springer Nature.

Figure 1.5 – Structure of the HCMV MIEP

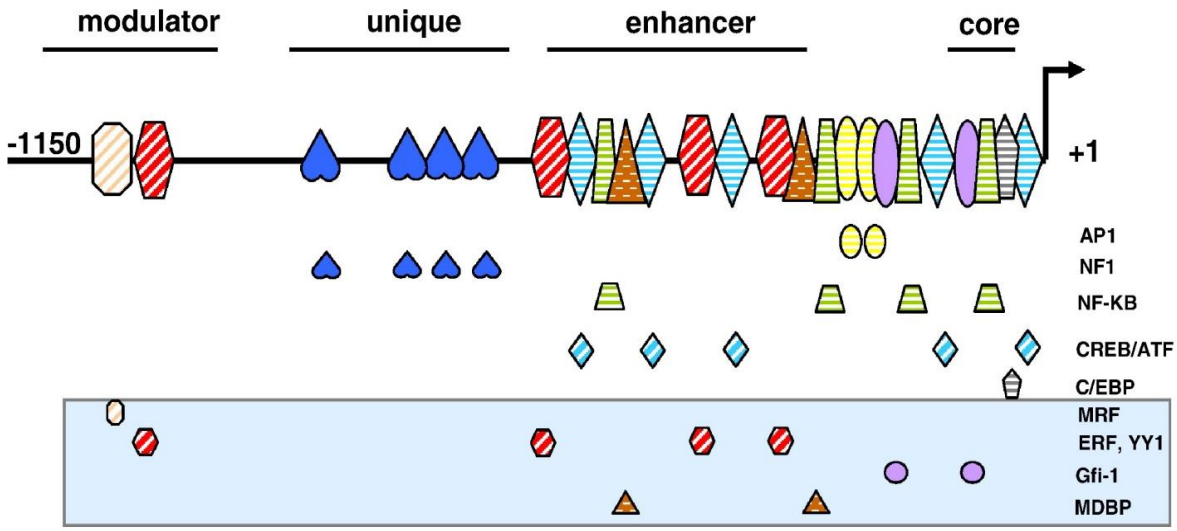


Figure 5. The HCMV MIEP comprises four distinct regions: a core promoter, an enhancer, a unique and a modulator region. Within the enhancer, a number of binding sites for known cellular transcription factors have been identified, enabling the region to be modulated by host mechanisms of gene regulation. NF-κB, CREB/ATF and YY1/ERF bind to the 18 bp, 19 bp and 21 bp repeats, respectively. The transcription start site is designated by the forward arrow at + 1. Negative regulatory factors are highlighted by the blue shaded background. Reproduced with permission from Sinclair and Sissons (1996).

Over the last several years, it has become increasingly clear that the chromatin landscape surrounding the MIEP plays a fundamental role in determining the transcriptional output of IE genes following signal transduction[165]. This is explained by the fact that regulators of the MIEP facilitate transcriptional activation / repression by recruiting co-factors involved in the post-translational modification of histone proteins, an epigenetic component integral to the higher-order molecular structure inherent to both host genomes and viral episomes[33,166]. As a result, it has been shown that as virally infected cells acquire a more permissive phenotype (e.g. during reactivation from latency), corresponding changes in histone modification patterns are observed at the MIEP, suggesting the adoption of a more "open" chromatin conformation that favours the activation of lytic MIE genes[167]. Moreover, when human fibroblasts are pre-emptively treated with histone deacetylase inhibitors, such as trichostatin A (TSA), this appears to counteract the effects of negative regulators of viral transcription, resulting in a loss of repressive histone marks that correlate with increased levels of IE gene expression[168,169]. Yet, though these findings are important in establishing the relationship between chromatin structure and regulation of viral transcriptional activity, it remains noteworthy that evidence of chromatin-mediated control – particularly at an early

stage of infection – points to the likely involvement of intrinsic antiviral immunity, in which the negative regulation of viral IE genes offers the potential to restrict productive replication[170]. Thus, given that the MIEP appears to be targeted for initial repression upon infection, this raises the question as to how the virus may seek to counteract such an early response in order to enable lytic replication to occur.

To account for the broad mechanism of intrinsic antiviral immunity, it is first necessary to consider the impact of nuclear events that place during the 'pre-IE' phase of HCMV infection (i.e. prior to the *de novo* expression of viral gene products, but immediately following cell entry). Although this broadly entails the rapid chromatinisation and circularisation of incoming viral DNA, of considerable importance is the fact that a vast majority of viral episomes come to form a close association with distinct nuclear complexes, previously referred to as ND10[171]. These are highly dynamic clusters of protein found throughout the host cell nucleus, which are generally defined by the presence of core constituents PML, hDaxx and Sp100, but have also been known to contain up to 70 other different proteins (Figure 1.6)[172]. A vast majority of ND10 components are post-translationally modified by conjugation to small ubiquitin-like modifier (SUMO) moieties and it is widely understood that the functionality of ND10 are highly dependent on nature of these modifications[173]. In uninfected cells, for instance, SUMOylated forms of PML serve as the main scaffold protein for ND10, particularly as these are intimately involved in the assembly and maintenance of these structures as well as the recruitment of other ND10-associated proteins, such as hDaxx[174–176]. Though ND10 have been implicated in a number of cellular processes, including apoptosis, tumour suppression as well as senescence, they share a complex relationship with various DNA viruses (including herpesviruses, adenoviruses, and papovaviruses)[177,178]. With respect to HCMV, it was initially hypothesised that these structures were essential for viral replication since initiation of transcription was only found to occur at sites where viral DNA had become deposited to ND10[179,180]. However, there is now strong evidence that ND10 manifest themselves as part of an innate defence against HCMV, along with other herpesviruses, specifically, by working against the onset of IE gene expression[181]. As such, treatment of cells with interferon enhances the expression of major ND10 constituents, such as PML or Sp100, resulting in an increase in both the number and size of ND10 structures[182]. Furthermore, depletion of each of the above core components has been shown to augment levels of MIE gene transcription following infection with HCMV, which goes on to support their individual identities as host restriction factors[183–186]. Of these, hDaxx remains one of the most studied in the context of lytic infection, and it has been shown that overexpression of this

factor in normally permissive cells renders them refractory to HCMV infection, whereas downregulation of hDaxx increases IE gene expression and subsequent virus propagation[187,188]. In addition, knockdown of hDaxx has been correlated with changes in chromatin structure at the MIEP, arguing for the ability of ND10 to impart its repressive effects through epigenetic modifications[185]. This finding appears to be linked to the ability of hDaxx to recruit ATRX, a known chromatin remodeler, where both form a complex that becomes sequestered to ND10[186,189]. Yet, it remains unclear as to whether PML or Sp100 may exert similar regulatory effects despite reports that these factors are also able to interact with other chromatin-modifying enzymes, such as histone deacetylases (HDACs) and methyltransferases (HMTs) as well as heterochromatin protein 1 (HP-1), all of which exhibit transcriptionally repressive properties[190–192].

Figure 1.6 – Components of ND10 structures

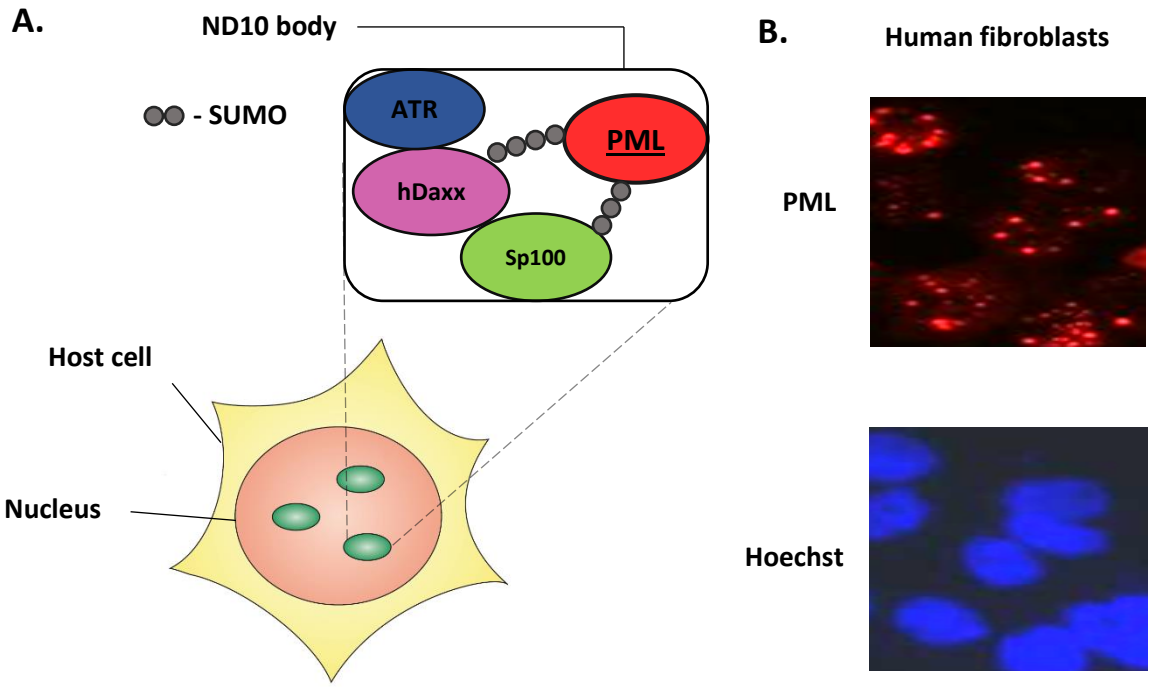


Figure 6. **A.** ND10 structures exist as discrete nuclear foci in most mammalian cells. They range from 0.2–1.0 μm in diameter and number between 1-30 bodies per nucleus, depending on the cell type, cell-cycle phase and stage of differentiation. ND10 are structurally and functionally heterogeneous, despite their uniform appearance. PML protein is necessary for the formation of ND10 and is heavily post-translationally modified. SUMOylation of PML is essential for ND10 integrity and enables PML to interact with several other ND10 constituents, namely Sp100 and hDaxx, which recruits ATRX. Combined, these factors contribute to the intrinsic antiviral function of ND10. **B.** ND10 accumulations in uninfected human fibroblasts; cells stained for PML (red) and nuclei (blue)

From the virus' perspective, a corollary to the presence of intrinsic defence mechanisms is the evolution of effective viral-based countermeasures. Naturally, therefore, most, if not all, herpesviruses possess the ability to overcome the antiviral restriction mediated by ND10 and encode a number of functionally diverse regulatory proteins to fulfil this role[178]. For herpes simplex virus type 1 (HSV-1), one of its IE gene products, ICP0, counteracts the effect of the repressive hDaxx /ATRX complex by inhibiting the SUMO interaction motif (SIM)-dependent recruitment of hDaxx to PML, and thence to the parental viral genome itself[193,194]. Furthermore, ICP0 also exhibits an E3 ubiquitin ligase activity that allows it to induce the proteasome-dependent degradation of SUMOylated PML, which is required for ND10 formation and consequent inhibitory effects on the virus[195,196]. In the case of HCMV, a closely related outcome of ND10 disruption is achieved by a different set of mechanisms involving two separate proteins. At the earliest stage of lytic infection, the tegument-derived pp71 protein is responsible for alleviating the effects of hDaxx/ATRX-mediated repression, which as noted above, is responsible for silencing the MIEP. Briefly, upon its successful translocation to the nucleus, pp71 is posited to displace ATRX from ND10 as well as target hDaxx for degradation through a proteasome-dependent, but ubiquitin-independent pathway[186,197]. Given that pp71-mediated disruption of hDaxx/ATRX remains critical for robust MIEP activation and IE gene expression at the onset of lytic infection, it is thus of additional note that the HCMV IE1 protein itself also participates in neutralising the repressive effects of ND10. By contrast to pp71, however, IE1 is able to induce the dispersal of entire ND10 structures, resulting in the complete displacement of ND10-associated proteins[198] . To account for this, studies have shown that IE1 promotes the loss of SUMOylated PML, akin to the function of HSV-1 ICP0[199]. Similarly, IE1 has also been shown to promote the loss of SUMOylated Sp100 proteins[184]. Though, interestingly, IE1 does not appear to possess any intrinsic ubiquitin ligase or SUMO protease (deSUMOylase) activity, giving rise to the notion that it may perhaps function by recruiting other cellular factors to remove SUMO from PML or Sp100 by preventing each of their respective isomerisation through an as yet undefined mechanism[200,201]. Importantly, consistent with the roles of pp71 and IE1 in separately counteracting the antiviral effects of hDaxx/ATRX and PML, respectively, HCMV inefficiently enters productive infection in the absence of pp71 or IE1 unless hDaxx/ATRX or PML are depleted prior to infection[187,202,203]. Furthermore, the finding that knockdown of hDaxx in combination with PML leads to a further increase in the efficiency of wild type HCMV replication, also provides additional evidence for the independent involvement of each of these factors in restricting HCMV[204]. Moreover, since Sp100 has been characterised as one of the first ND10-related factors to exhibit the potential to inhibit HCMV replication at a late

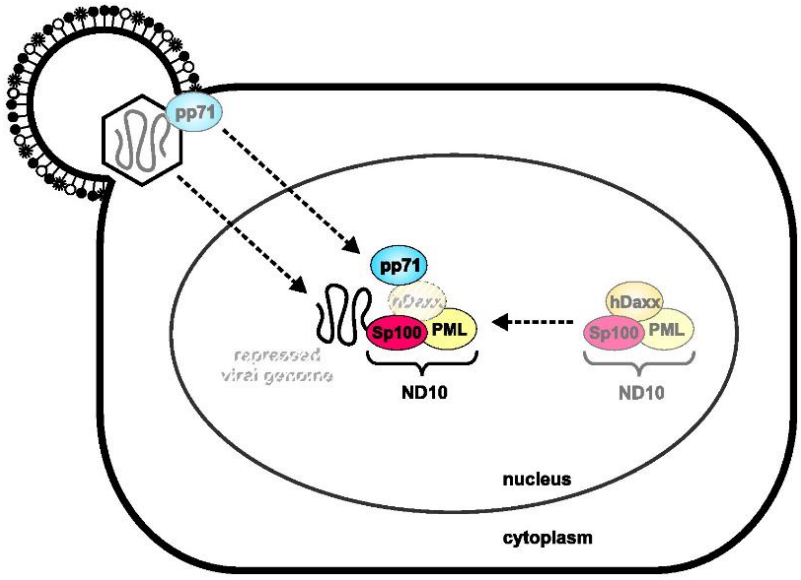
stage of infection, it is noteworthy that IE1 may also help to induce the proteasome-dependent degradation of unSUMOylated Sp100 during this period; however, the exact mechanism by which it does this remains unclear[202].

Taken together, this information may be used to create a working model that describes the crucial early events which occur during a typical HCMV infection in permissive cell types: (i) immediately following cell entry, parental viral genomes become targeted by ND10 in the nucleus, resulting in the formation of repressive chromatin around the viral DNA; (ii) once viral tegument-delivered pp71 is imported into the nucleus, it antagonises hDaxx/ATRX-related repression, which then permits the initiation of viral IE gene expression; (iii) the expression and accumulation of high levels of IE1 helps to subsequently overcome PML-mediated suppression, which in turn induces the dispersal of ND10; (iv) the combined modification of ND10 relieves the broad effects of transcriptional repression on viral genomes deposited in the nucleus, which subsequently correlates with efficient lytic infection (Figure 1.7).

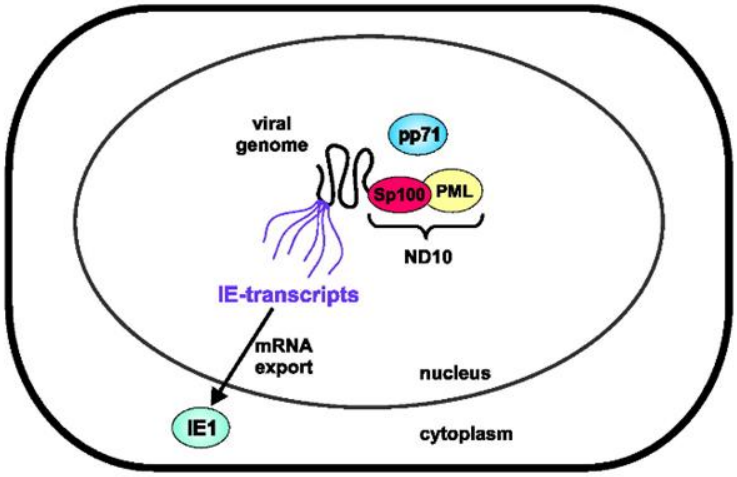
Figure 1.7 – Interplay of HCMV and ND10 components during lytic infection

A.

In cells permissive for productive HCMV infection, viral pp71 protein is capable of entering the host nucleus in order to target hDaxx for degradation.



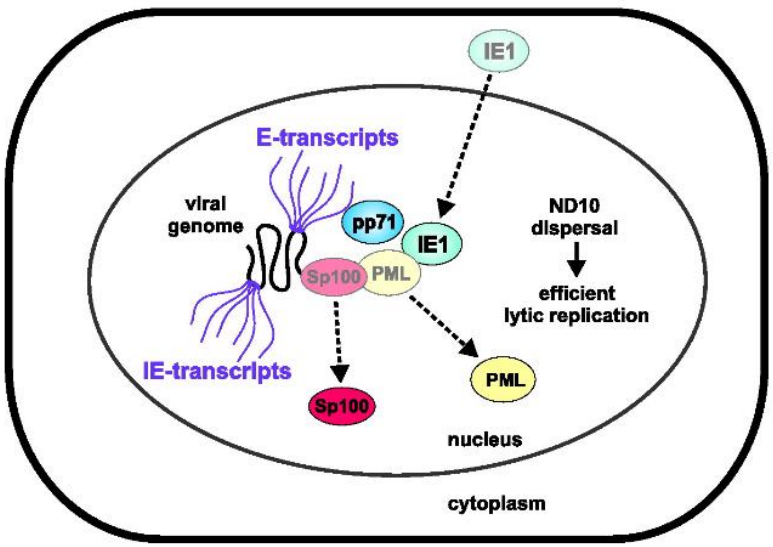
B.



pp71-induced reduction of hDaxx relieves repression of the viral chromatin, which leads to initiation of viral IE gene expression and production of the key lytic viral transactivator IE1.

C.

IE1 localises to ND10 to promote the complete disruption of this structure. ND10 dispersal by IE1 correlates with efficient lytic viral replication. Reproduced from Tavalai and Stamminger (2008) with permission from Elsevier.



1.4. HCMV: LATENCY

Strategies that limit the possibility of eradication following primary infection are collectively employed by herpesviruses to promote lifelong persistence within the infected host. Most notable among these is the hallmark ability of herpesviruses to enter a latent state of infection, insofar as adopting a quiescent phenotype. Not only does this benefit the virus from avoiding clearance by the host immune system, it nonetheless leads to the establishment of latent viral reservoir that has the recognised potential to spread and recrudesce over time.

Viral latency, by way of preamble, may be operationally defined as the retention of virus genome in the absence of infectious virion production – which, upon appropriate stimulation, can be reversed to promote reactivation followed by lytic replication[205]. While this definition does not preclude the possibility of an array of viral gene transcripts from becoming expressed during the latent period, it nonetheless distinguishes latency from an otherwise persistent viral infection involving low levels of ongoing productive replication (cf. patients with chronic Hepatitis C).

During latent infection, herpesviruses exhibit a number of common phenotypic features. The relative lack of lytic viral gene expression is by far the most crucial, but other aspects of note include the maintenance of virus genome in the form of nuclear episomes (with the notable exception of HHV-6) and the restriction of latency to specific cellular sites in the body. Knowledge of these distinct properties grant insight into the essential processes that govern herpesvirus latency, which are complex, but very closely interlinked, and above all, responsible for conferring control over virus gene expression.

1.4.1. Current Model of HCMV Latency in the Context of Natural Infection

Prior to the advent of PCR-based technology, attempts to define the location(s) of latent carriage of HCMV were hindered by an inability to detect viral genomes in seropositive individuals. Nonetheless, that at least one major site of latency was likely to be found in the peripheral blood compartment came from early clinical observations in which leukocyte-depleted blood products could significantly reduce the rate of HCMV transmission from asymptomatic carriers[206–208]. Current evidence now supports this view, in that,

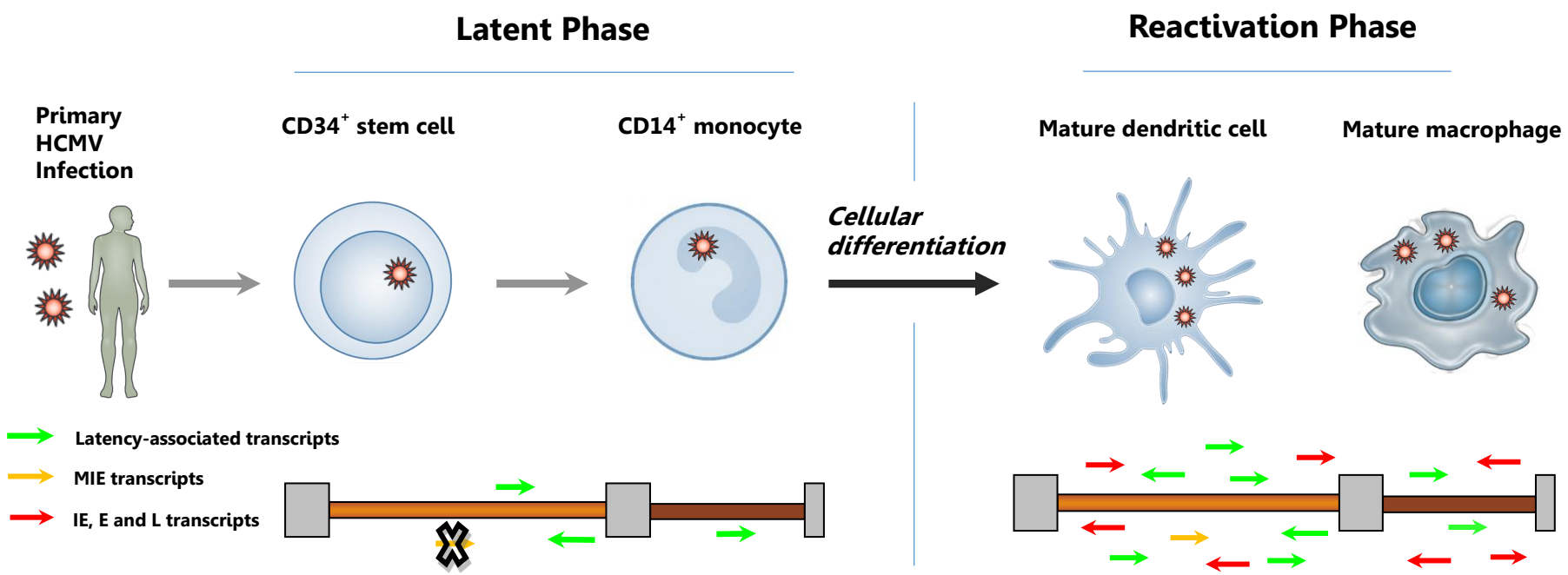
specifically, cells of the early myeloid lineage represent sites of natural latency *in vivo*[209]. Through the use of more sensitive assays, the presence of latent viral genomes have been detected in peripheral blood monocytes and granulocyte-macrophage precursors as well as $CD34^+$ haematopoietic stem cells resident in the bone marrow[95,210,211]. These results have since been recapitulated under *in vitro* conditions, supporting the general view that myeloid progenitor cells, including their monocytic derivatives, are not susceptible to productive HCMV infection and are able to maintain viral genomes in the absence of lytic gene expression[103,212,213]. Interestingly, members of the lymphoid lineage of haematopoiesis (i.e. peripheral blood B or T cells) do not appear to harbour latent viral genomes, despite them also originating from $CD34^+$ progenitors[95,214]. How such lineage specificity may be exhibited on the part of latent carriage remains unclear, but one suggestion is that latent infection itself may help to promote commitment to the myeloid lineage[215]. Nonetheless, these results do not rule out other sites of latency, which remain the focus of further research[216–218].

As lineage commitment progresses, however, myeloid cells cease being able to maintain HCMV in a latent state, and instead, become associated with viral reactivation from latency. The differentiation of myeloid progenitors to macrophage-like cells or dendritic cells has been shown to trigger reactivation in natural and experimental systems of HCMV latency, leading to the expression of all classes of lytic viral genes and the production of infectious virions[219,220]. Such a phenomenon is consistent with the finding that terminally differentiated myeloid cells are fully permissive for productive HCMV infection, like that of infected fibroblasts[221,222]. These results thus lend credence to the notion that myeloid cells act conjointly as sites of HCMV latency as well as reactivation *in vivo*, with the state of cellular differentiation serving as the crucial link between the two events[209] (Figure 1.8). In line with this, recent analysis of clinical samples taken directly from HCMV-seropositive individuals has revealed that late-differentiated myeloid cell types (i.e. circulating dendritic cells and alveolar macrophages) undergo clear lytic gene expression and are capable of supporting viral replication following culture *in vitro*[167,223].

Because latent viral genomes are carried by a very small population of naturally infected cells (approximately 0.004–0.01% of monocytes, at around 2–13 genome copies per cell), it is not surprising that several model systems of HCMV latency have been developed to complement the study of its related mechanisms[224]. For instance, models of experimental latency, which make use of precursor cell lines (e.g. myelomonocytic THP-1 and teratocarcinoma NT2D1 cell lines), are known for their intrinsic ability to block the expression of viral lytic genes and

thereby support latency, except upon differentiation[225,226]. These *in vitro* systems have been instrumental in identifying the cellular factors that modulate latent HCMV infection, even though they only provide a snapshot of the virus life cycle as they are unable to fully reactivate latent viral genomes and participate in new virion production[227–234]. By contrast, *ex vivo* models of latency, which are based on the infection of primary myeloid cells, provide an arguably more complete view of HCMV latency, given that these are able to recapitulate the production of infectious virus as the defining characteristic of viral reactivation[235–237]. Though, in utilising these more robust systems, one must take into account the limitations of primary cell culture, notably isolation costs, coupled with finite numbers of cells, reduced life spans *ex vivo*, and implicit donor variation. These negative aspects make them impractical for certain methodological applications, such as the generation of stable cell lines with which to employ molecular biology techniques for manipulating gene expression. Yet, as there has not been a suitable animal model for HCMV, clinical evidence remains critical for arriving at conclusions that are material to the *in vivo* situation; however, *in vitro* analysis still has the potential to offer important insight[238]. Indeed, experimental research into the mechanisms regulating HCMV latency has led to the discovery of novel extracellular stimuli that can promote viral reactivation following treatment, either, by triggering cellular differentiation (akin to conventional cytokine stimulation) in the case of vitamin D3 or by directly activating lytic gene expression as with dexamethasone[239,240]. These reagents can be used as tools to promote the study of HCMV in model systems without necessarily compromising their capacity to reflect the natural course of infection.

Figure 1.8 – Current model of HCMV latency and reactivation in the myeloid linage of haematopoiesis



Following primary HCMV infection, the virus establishes a lifelong latent infection in $CD34^+$ stem cells residing in the bone marrow as well as circulating $CD14^+$ monocytes. Viral latency is characterised by the maintenance of virus genome in the concomitant absence of infectious virion production. During this period, a number of HCMV latency-associated genes become expressed (green arrows), but there is restricted expression of lytic viral genes. This is underscored by chromatin-mediated repression of the viral MIE genes (yellow arrow with black cross), which otherwise serve as the main viral transactivators. Once these immature cell types have undergone terminal differentiation, however, to become either mature dendritic cells or macrophages, the latent viruses become triggered to reactivate, resulting in the detectable expression of MIE genes, along with all other lytic temporal classes (red arrows) and the subsequent production of new infectious virions.

1.4.1. Regulation of HCMV Latency

One of the most crucial elements in regards to HCMV latency concerns the specific control of viral IE gene expression, particularly as the induction of these genes is inseparable from the otherwise divergent lytic temporal cascade. As such, the MIEP (which is responsible for directing the expression of major viral transactivators IE1 and IE2) appears to be subjected to a differentiation-dependent transcriptional regulation, thereby reflecting the relationship between the differentiation status of the host cell and the permissiveness for HCMV to undergo productive replication[209]. Indeed, binding sites for multiple cellular transcription factors have been located within the MIEP, and it has been posited that the relative balance of these factors in a given cell type serves as an upstream determinant of HCMV IE gene expression[163]. During latency, it is likely that this balance favours interactions with known co-repressor complexes, made of up host chromatin remodelers which help to catalyse the local formation of repressive histone modifications and aid in nucleosome compaction[165]. So far, the nuclear transcription factors Yin Yang 1 (YY1), Ets-2 repressor factor (ERF), growth factor independent 1, and more recently, KRAB-associated protein 1 (KAP1) have all been shown to act as negative regulators of the MIEP in non-permissive cells, where they facilitate the local recruitment of HDACs and HMTs that go on to repress its activity[241–244]. As referred to earlier, nucleosome occupancy in tandem with post-translational histone modifications have been intimately linked to the timing and extent of transcription from the MIEP, revealing themselves to be critical in influencing the outcome of not just lytic, but also latent infections. Accordingly, under latent conditions, the MIEP has been found to be maintained in a repressed chromatin state, as demonstrated by its association with reduced levels of histone H4 acetylation as well as increased levels of histone H3 trimethylation at lysine 9 (H3K9me3) and 27 (H3K9me27)[167,245]. The latter of these serve as targets for recruitment of HP-1 and Polycomb group proteins, respectively, which are well established to play key roles in augmenting transcriptional silencing[230,246]. Importantly, the detection of IE gene transcription is consistent with these histone modification patterns, with *in vitro* models of latency exhibiting weak and transient expression of IE1, accompanied by undetectable levels of IE2, while in a similar manner, latent infection of primary myeloid cells displaying the sporadic expression of both genes[247,248]. Notably, the recent observation that treatment of latently infected cells with histone deacetylase inhibitors results in a transient induction of IE gene expression is highly supportive of a role for chromatin structure in regulating the MIEP during latency[249]. It is only upon terminal cellular differentiation – which triggers viral reactivation – that a robust expression of IE genes is observed and the chromatin

configuration at the MIEP becomes subsequently altered, incurring changes such as a loss of HP-1 and an increase in levels of histone acetylation[167].

Despite the fact that regulation of viral IE gene expression remains critical for HCMV latency, there is now strong evidence demonstrating that latent infection requires more than just silencing of lytic gene expression and that efficient latent carriage is associated with the ability of HCMV to efficiently undergo viral gene expression[247]. Indeed, clear differences in the epigenetic landscape across the virus genome have been detected in latently infected cells, supporting the existence of viral gene transcription throughout this period[245]. Thus, during latent infection, it has been shown that HCMV incorporates a programme of gene transcription, one which does not fall into a regulated cascade as that of a lytic infection and is otherwise severely restricted in its overall degree of expression[250–252]. Yet, the general properties that characterise latency (i.e. low levels of viral genome carriage and corresponding gene expression) have made it difficult to understand molecularly. Until recently, the latent transcription programme was presumed to be limited to a discrete number of viral RNAs and proteins, as chiefly defined by compiled microarray data. However, global transcriptome analysis of HCMV latency – facilitated by the application of next-generation sequencing (NGS) techniques – indicates that the extent of viral gene expression during latent carriage may be far wider than first thought[245,253]. Additionally, when a single-cell approach is used, it appears that there may be stages of latency establishment which eventually result in a latent transcriptome with some qualitative changes (e.g. a lack of major IE gene expression) but mainly quantitative differences in viral gene expression, one that bears some form of resemblance to a lytic state of infection[254]. In spite of several caveats accompanying these interpretations, most notably the difficulties in safeguarding the potentiality of unbiased reads from lytic transcriptional noise, it is now clear that the notion of strict quiescence during viral latency is being challenged such that it may be far more complicated than previously appreciated[255]. Nevertheless, this has not diminished the importance of ongoing investigations towards the identification and functional characterisation of individual latently expressed transcripts, on grounds that they are likely to serve important roles in mediating latent HCMV infection[256]. Consequently, a number of distinct viral gene regions or ORFs have been defined as being transcriptionally active during latency, with the products of these ORFs being divided into two separate groups of either: cytomegalovirus latency-specific transcripts (CLTs), which denote spliced and unspliced RNAs sourced from both strands of the MIE region, or other so-called LATs, which are encoded by the rest of the genome and found to be expressed during both latent and lytic phases of infection (albeit with their expression during lytic infection

being IE gene dependent)[209]. Below, Table 1.3 outlines the identities and known functions of a number of these latently expressed transcripts.

Table 1.3 – Gene products and functions during latency and lytic infection [Adapted from: Sinclair and Reeves. Viruses (2013)]

Gene Product	Latent Function	Lytic Function	Ref.
Cytomegalovirus latency-associated transcripts (CLTs)	Unknown	Regulation of antiviral 2'5' OAS expression (ORF94)	251,252,257,258
UL138	Regulation of RNFRI and MRP1, repression of the MIEP	Regulation of TNFRI and MRP1, virus maturation (via 133-138 locus)	259–262
UL81-82ast	Promotes UL138 gene expression	Unknown	263–266
LAcmvIL-10	Down-regulation of MHC class II expression, immune evasion	Unknown – cmvIL-10 is expressed during lytic infection	267,268
Lnc4.9	Binds Polycomb repressor complex 2, silencing of the MIEP	Unknown	245
UL84	Genome maintenance	DNA replication, UTPase activity, transcriptional regulation	42,269–271
US28	Attenuates MAPK and NF-κB signalling, maintenance of latency	GPCR, induces cell signalling and migration, agonist of the MIEP	249,272–279
UL144	Unknown	TNF superfamily member, hijacking of NF-κB signalling, immune response modulation	280–283

Although the full extent of the latent transcriptome and its properties remain to be elucidated, it is nonetheless clear that the successful establishment and maintenance of HCMV latency is dependent on the expression of latent viral genes. This stems principally from the fact that manipulation of the cell by the virus is required to confer conditions necessary to support latency[284]. Here, the specific functions of a subset of LATs, namely UL138, UL144, viral interleukin-10 (latency-associated CMV homologue of IL-10 [LAcmvIL-10]) and IE1x4 will be discussed further.

To start with, UL138 was the first viral gene product to be characterised as an important determinant of latent infection. Recombinant viruses lacking UL138 were found to be unable to establish latency in primary myeloid cells, showing commensurate levels of productive replication[260]. One possible mechanism by which UL138 may promote latency is by maintaining the MIEP in a transcriptionally repressed state. This is backed up by recent evidence demonstrating that expression of UL138 inhibits the local recruitment of histone demethylases, thereby limiting the potential for the repressed chromatin signature found at the MIEP to become reversed[285]. Although the UL138 gene appears to encode a 21-kDa type-1 transmembrane protein that localises to the Golgi apparatus, how it precisely functions remains unclear[260]. One hypothesis posits an involvement in the vesicular trafficking of host cell receptors, given that UL138 expression is associated with decreased surface levels of multidrug resistance-associated protein 1 (MRP1) – which incidentally improves the killing of latently infected cells by vincristine – but increased levels of tumour necrosis factor receptor 1 (TNFR1) and epidermal growth factor receptor (EGFR). In the case of EGFR, activation of its associated downstream signalling pathways (i.e. PI3K and ERK/MAPK) has been shown to enhance survival of the latently infected cell by inhibiting caspase 3 activation. Consequently, it is also possible that signalling events stemming from such UL138-mediated regulation of EGFR or indeed other receptors might be responsible for precipitating changes in epigenetic silencing. Notably, because the UL138 gene is located in the ULb' region of the virus genome, its expression is naturally inherent to low-passage clinical isolates. Yet, interestingly, studies performed using laboratory-adapted strains – which do not possess the ULb' region – have indicated that the ULb' is not essential for latency establishment *in vitro*, calling into question the role of UL138 in the latently infected cell[259]. Since low-passage strains are considerably more robust than their laboratory-adapted counterparts in establishing latency, this has led to the general assumption that UL138, along with other ULb' genes – known also to be latently expressed – are collectively involved in establishing latent infection, and current evidence supports this view[286].

Like UL138, the UL144 ORF is also located in the ULb' region of the genome. Though not required for latency establishment, it has however been implicated in promoting optimal latency, supporting the view that multiple UL/b′ genes contribute towards this process[259]. Specifically, UL144 encodes a transmembrane protein that is a structural homologue of the herpesvirus entry mediator HVEM[283]. Under normal conditions, HVEM functions as part of the signalling network HVEM-LIGHT-BTLA, which is broadly implicated in orchestrating inflammation and homeostasis of lymphoid microenvironments[287]. In this respect, UL144

engages with the HVEM ligand, BTLA, initiating an inhibitory signalling response that downregulates T cell proliferation[288]. Thus, it has been proposed that HCMV may use UL144 to inhibit T cell immune responses in order to prevent clearance of the virus. Indeed, despite clinical isolates of HCMV exhibiting clear sequence variation in the ectodomain of UL144, all are known to retain selective binding to BTLA, but not other ligands, such as TNF-α and LIGHT (a known antagonist to BTLA)[289]. This argues for significant immunological pressure in shaping the evolution of this molecule. Additionally, the intracellular domain of UL144 is understood to signal via NF-κB, TRAF6 and TRIM23, which leads to the induction of the chemokine CCL22, a known T_H2 chemoattractant that can help subvert the T_H1 immune response[280,281,290]. Nonetheless, whether such signalling through this pathway is important for latency remains unclear.

Homologues of the host immunomodulatory cytokine interleukin-10 (IL-10) are expressed by HCMV, comprising two major isoforms encoded within the *UL111A* gene region. During lytic infection, *UL111A* is expressed as a doubly spliced transcript, denoted as cmvIL-10[291]. However, under latent conditions, a latency-associated form of cmvIL-10, termed LAcmvIL-10, is also transcribed[267]. Despite their close sequence similarity, which includes shared usage of the same translation initiation site, both transcripts undergo differential splicing that results in LAcmvIL-10 retaining one of two introns found in cmvIL-10[267]. In terms of function, like its nearest counterpart, LAcmvIL-10 demonstrates a clear ability to downmodulate the activities of MHC class II presentation in early myeloid cells[292]. As such, expression of recombinant LAcmvIL-10 has been associated with reduced delivery of MHC class II to the cell surface as well as reduced levels of transcription of MHC class II components, such as invariant chain[268]. Though, in contrast to cmvIL-10, LAcmvIL-10 is unable to exert an immunosuppressive effect on DC maturation, which is thought to be linked to the inability of LAcmvIL-10 to bind to the human IL-10 receptor[293]. Nevertheless, in the context of latent infection, LAcmvIL-10 has been shown to render cells refractory to $CD4^+$ cell recognition, thus implicating its role in helping to maintain latent viral carriage in otherwise healthy, immunocompetent individuals[294].

IE exon 4 (IE1x4) is a recently identified LAT that is reported to be encoded by exon 4 of IE72. Despite its clear association with lytic gene expression, Tarrant-Elorza *et al*. observed that IE1x4 could be expressed in latently infected $CD34^+$ cells as a discrete gene product[295]. The protein was shown to tether plasmids containing HCMV maintenance elements to cellular chromatin through interactions with host topoisomerase IIB and DNA binding protein Sp1,

revealing a possible mechanism for latent viral genome carriage to be maintained during cell division. This finding strengthens the argument for genome replication during latency, which while known to be generally inefficient and not consistently documented throughout the literature, may otherwise be able to sustain the pool of latently infected cells *in vivo*[296].

1.4.1. UL81-82 antisense transcript / LUNA

The UL81-82 antisense transcript (UL81-82ast) is a latently expressed viral gene product encoded by the UL81-82 locus of the HCMV genome. It was originally discovered by Bego *et al.*, who observed that transcription of this mRNA originated from the opposite strand encoding the UL81 gene while being partially antisense to UL82[264]. The transcript give rises to a putative 133 amino acid serine-rich protein, termed LUNA (Latency Unique Nuclear Antigen), which appears to be highly conserved across both clinical- and laboratory-derived strains of HCMV as well as chimpanzee CMV. The protein has been transiently detected in human fibroblasts early upon lytic infection, but it is nonetheless considered to be latency-associated, owing to its stable expression under latent conditions *in vitro*[263]. As such, its presence has been confirmed in primary monocytes and $CD34^+$ myeloid progenitor cells isolated from healthy HCMV-seropositive donors in the absence of lytic viral gene expression[265]. In certain cases, the detection of LUNA-specific antibodies in the sera of other such carriers provides strong evidence that LUNA is also able to be produced *in vivo* and has the capacity to elicit an immune response[297].

To date, only a few attempts have been made to characterise the role of the LUNA protein during HCMV latency. While no precise function has yet been attributed to it in natural latency, studies on experimental latency have nonetheless been informative, implicating the protein in latent carriage and viral reactivation. In a study devised by Keyes *et al.*, the function of LUNA in HCMV latency was evaluated through the use of a LUNA deficient virus that had been generated by BAC mutagenesis in the FIX strain backbone[266]. The impact of this FIX-ΔLUNA virus was explored in various models of infection that reflected settings of permissiveness for productive infection or latency establishment. Notably, FIX-ΔLUNA was able to undergo productive infection in fibroblasts, indicating that LUNA was dispensable for lytic replication. However, latently infected $CD14^+$ monocytes carrying FIX-ΔLUNA failed to express detectable amounts of the UL138 latency-associated transcript, which given its requirement for latency establishment, suggested that LUNA was important in promoting

efficient UL138 expression in order to mediate this process. Moreover, FIX-ΔLUNA virus failed to reactivate from infected $CD14^+$ cells following differentiation.

1.5. Aims and objectives

The principal focus of this research is to improve our understanding of the general mechanisms that underlie HCMV latency. As outlined earlier, the expression of latency-associated viral gene products appears to be important for latent HCMV infections, warranting their use for further study. Thus, to achieve the stated objective, an investigation will be carried out to evaluate the functional significance of LUNA, an example of one such viral gene product known to be expressed during latency.

To the extent that LUNA is implicated in regulating HCMV latency, its expression has already been associated with the efficient transcription of UL138, which may be important for the proper establishment of latent infection. Indeed, in this context, LUNA is also required for the reactivation of latently infected cells. Assuming that the augmentation of latency-associated virus gene expression is necessary for latent viral genomes to remain responsive to appropriate environmental cues, one may hypothesise that LUNA is specifically involved in controlling viral gene expression during latency.

2. Materials and Methods

2.1. MATERIALS

2.1.1. Solutions and buffers

Complete media: RPMI-20

- RPMI-1640 (Sigma-Aldrich)
- 20 % (v/v) Foetal Calf Serum (FCS)
- 1 % Streptomycin (Pen/Strep)

DMEM-10

- DMEM (Life Technologies Ltd.)
- 10 % (v/v) FCS
- Pen/Strep

Haematopoietic Media

- X-Vivo 15 with Gentamicin, L-glutamine and phenol red (Lonza)

Freeze mix: 90 % FCS: 10 % dimethyl sulfoxide (DMSO)

TE buffer: 10mM Tris pH 8.0
1mM EDTA

Magnetic separation buffer: 1 × Phosphate Buffered Saline (PBS) (Lonza)
2 mM EDTA (Sigma-Aldrich)
0.5 % (v/v) FCS

2 × Laemmli sample buffer: 0.125 M Tris-HCL, pH 6.8
20 % (v/v) glycerol
4 % (v/v) SDS
1.25 % (v/v) 2-Mercaptoethanol
0.004 % (v/v) bromophenol blue

SDS-PAGE buffer:	3 g/L Tris-base
	14.4 g/L glycine
	1 g/L SDS
	ddH_2O to 1 litre
Transfer buffer:	1 g/L Tris-base
	4.8 g/L glycine
	20 % (v/v) methanol
	ddH_2O to 1 litre
12 % Resolving gel:	10 mL acrylamide
	3.75 mL 0. 5M Tris, pH 8.8
	0.3 mL 10 % SDS
	16.95 mL dH_2O
	15 µL TEMED
	1 mL 1.5 % ammonium persulphate
12 % Stacking gel:	2.5 mL acrylamide
	5 mL 0.5 M Tris, pH 6.8
	0.2 mL 10 % SDS
	11.3 mL dH_2O
	15 µL TEMED
	1 mL 1.5 % ammonium persulphate
ChIP cell lysis buffer:	50 mM Herpes pH 7.9
	140 mM NaCl
	1 mM EDTA pH 8.0
	10 % (v/v) glycerol
	0.5 % (v/v) NP-40
	0.25 % (v/v) Triton X-100
	1 × cOmplete™ EDTA-free protease inhibitor cocktail (Roche)
ChIP wash buffer:	10 mM Tris-HCl pH 8.0
	200 mM NaCl
	1 mM EDTA pH 8.0
	1× cOmplete™ EDTA-free protease inhibitor cocktail

Shearing buffer:	0.1 % w/v SDS
	1 mM EDTA
	10 mM Tris-HCl pH 8.0
	1 × cOmplete™ EDTA-free protease inhibitor cocktail
IP Dilution buffer:	20 mM Tris pH 8.1
	2 mM EDTA
	150 mM NaCl
	1 % Triton X-100
	0.01 % SDS
	1 × cOmplete™ EDTA-free protease inhibitor cocktail
IP Wash buffer 1:	20 mM Tris pH 8.1
	2 mM EDTA
	50 mM NaCl
	1 % Triton X-100
	0.1 % SDS
IP Wash buffer 2:	10 mM Tris pH 8.1
	1 mM EDTA
	0.25 M LiCl
	1 % sodium deoxycholate monohydrate
	1 % NP-40
Elution buffer:	100 mM $NaHCO_3$
	1 % SDS
RIPA lysis buffer:	50 mM Tris pH 7.4
	1 % Nonidet P-40 (NP-40)
	150 mM NaCl
	0.25 % sodium deoxycholate monohydrate
	1 % NP-40
	1 × cOmplete™ EDTA-free protease inhibitor cocktail

2.1.2. Plasmids

Reporter plasmids containing viral promoter regions driving luciferase expression were based on pGL3 (Promega). These included pGL3-UL144 and pGL3-LUNA, and were kind gifts of Emma Poole (Department of Medicine, University of Cambridge). LUNA gene expression vectors were based on pCMV-Tag2b (Stratagene). Plasmids encoding for FLAG-tagged WT LUNA (pCMV-Tag2b-FLAG-LUNA) and FLAG-tagged LUNAg233c point mutant (pCMV-Tag2b-FLAG-g233c) were kindly provided by Emma Poole and Matthew Reeves (Institute of Immunity and Transplantation, University College London). To generate FLAG-tagged WT LUNA, the LUNA coding region was excised from a previously published PET102UL82as10 LUNA expression (BamHI/HindIII) casette and inserted directly into pCMV-Tag2b in-frame with the FLAG-encoding sequence[263]. To generate the FLAG-tagged LUNA g233c point mutant, a guanine-to-cytosine transition was introduced by site-directed mutagenesis at position 233 of the LUNA nucleotide sequence, substituting cysteine for an activate serine residue. Plasmid pEF-BOS-GATA-2 was a kind gift of B. Gottgens (Cambridge, United Kingdom).

2.1.3. HCMV strains

eGFP-expressing variants of low passage HCMV strain TB40E (BAC-derived viruses TB40-BAC4) were kindly gifted by Michael Nevels (University of St. Andrews, St. Andrews, United Kingdom). These included WT phenotypic revertant (*rv*IE1), IE1-deficient mutant (*dl*IE1) and IE1 exon 4 deletion mutant (*dl*IE1ex4) viruses. All were derived from a modified bacterial artificial chromosome (BAC) containing an SV40-eGFP-BGH PolyA cassette inserted between viral US34 and TRS1 genes. An IE2-RFP tagged HCMV TB40-BAC4 variant was a kind gift of Betty Lau (University of Cambridge, Cambridge, United Kingdom).

BAC recombineering in the clinical reference strain Merlin (pAL1174) containing a UL32-GFP fusion reporter was performed to generate wild-type phenotype and mutant viral constructs. These included Merlin (WT), LUNA translation mutant (LUNA Del) and LUNA functional / catalytically inactive mutant (g233c). For LUNA Del, a cloning strategy was adopted to introduce a guanine to adenine exchange at nucleotide position 118965, mutating the tryptophan (TGG) to a stop codon (TGA). Because the codon change on the anti-parallel strand (ACC → ACA) was redundant for threonine, it was possible to maintain the integrity of corresponding pp71. Similarly, for g233c, to disrupt the predicted SUMOylation activity

without affecting pp71, a guanine to cytosine nucleotide exchange at position 233 of the LUNA nucleotide sequence resulted in the replacement of cysteine for a serine residue at nucleotide position 119197. Following sequence verification, the recombinant BAC viruses were transfected into fibroblasts using lipofectamine 2000 according to the manufacturer's instructions in order to reconstitute infectious virus. A revertant virus (Rev) was constructed from the LUNA Del virus using the same cloning strategy, but an equivalent phenotype virus was not generated from g233c. As shown subsequently, the Rev virus was identical to the WT parental virus in growth kinetics in fibroblast cell systems and was used as the primary wildtype phenotype control virus in HCMV infection experiments.

2.2. PRIMARY CELL CULTURE

2.2.1. Ethics statement

Ethical permission for this study was granted by the Cambridgeshire 2 Research Ethics Committee in accordance with the Declaration of Helsinki (REC reference 97/092). Informed written consent was obtained from all volunteers prior to providing blood samples.

2.2.2. Human $CD14^+$ mononuclear cell preparation

Venous blood was obtained from healthy donors by venepuncture, performed by a qualified phlebotomist. To prevent clotting, blood was diluted 1:1 in PBS containing 10 U/mL heparin sodium salt (Wockhardt UK Ltd.). Ficoll-Hypaque density gradient centrifugation was performed by layering 25 mL blood solution on to 12.5 mL Lymphoprep (Axis-Shield) and spinning at 800 *g* for 15 mins with no brake. Peripheral blood mononuclear cells (PBMCs) were then extracted from the plasma-Ficoll interface using sterile Pasteur pipettes, before being washed twice and resuspended in sterile PBS (Sigma-Aldrich). Positive selection using antibody-conjugated MicroBeads (Miltenyi Biotec) allowed collection of a $CD14^+$ mononuclear cell-enriched population. Briefly, PBMCs were resuspended in magnetic separation buffer and incubated with 20 µL anti-CD14-coated microbeads per 10^7 cells for 20 mins at 4°C, before being layered on a MACS® LS column prewetted with 3ml magnetic separation buffer. Bound cells were then washed three times in 3mL magnetic separation buffer, before being eluted in 3ml PBS. The isolated mononuclear fraction was plated to a density of 2×10^6 cells in 100mm dishes for ChIP assays, 5×10^5 cells/well in 24-well plates for RT-qPCR analysis, or to 1×10^5 cells/well in 96-well plates for immunofluorescent staining, and then incubated at 37°C, 5 % CO_2 for 2-3 hours to promote adherence. The PBS

was removed and replaced with X-Vivo 15; 10 ml for 100 mm dishes, 1 ml per well for 24-well plates or 200 µL per well for 96-well plates.

2.2.3. Human CD34$^+$ myeloid progenitor cell preparation

Cryopreserved human bone marrow CD34$^+$ progenitors isolated from granulocyte-colony stimulating factor (GCSF) mobilised healthy donors were purchased from Lonza and resuscitated according to Lonza's 'Procedure for thawing Poietics™ cells' with modifications. Ampoules were rapidly thawed by direct immersion in a 37°C water bath and diluted into a large volume of pre-warmed thawing media [X-Vivo 15, supplemented with 1 % BSA and DNase I (Sigma) at 20 U/ml], added in a drop-wise manner with gentle swirling. Solutions were pelleted by centrifugation at 200 *g* for 15 minutes, after which cells were resuspended in 1×10^6 cells/ml in X-Vivo 15 and incubated overnight at 37°C, 5 % CO_2, before cell viability was assessed by exclusion of 0.4 % trypan blue solution (Sigma). Cells were then plated in 1ml per well for 24-well plates.

2.2.4. Terminal myeloid cell differentiation

To promote differentiation to a monocyte-derived dendritic cell phenotype, CD14$^+$ cell cultures were stimulated with granulocyte macrophage colony-stimulated factor (GMCSF; 100 ng/ml) and IL-4 (100 ng/ml) for 5 days (both cytokines Peprotech). Mature DCs were then generated by adding LPS in fresh media (50 ng/ml; Sigma-Aldrich) for 2 days. To promote differentiation to a Langerhans cell (LC) phenotype, CD34$^+$ cells were stimulated with transforming growth factor-beta (TGF-β; 500 ng/ml), Fms-related tyrosine kinase 3 ligand (Flt-3L; 100 ng/ml), GM-CSF (100 ng/ml), TNF-α (2.5 ng/ml) and stem cell factor (20 ng/ml) (all from Peprotech) and cultured for 7 days until formation of characteristic cell clusters was observed. Immature LCs were then stimulated with LPS (500 ng/ml) for 24 hours to facilitate maturation.

2.3. CELL CULTURE SYSTEMS *IN VITRO*

2.3.1. Fibroblast cell culture

Human foreskin fibroblasts (HFF) (ATCC® #SCRC-1041) were maintained in 175 cm^2 culture (T175) flasks in DMEM-10 and incubated at 37°C in a humidified 5 % CO_2

atmosphere (37°C, 5 % CO_2). Confluent cultures were passed every 4-5 days by washing cells in phosphate buffer solution (PBS) and adding 4 mL Trypsin-EDTA (0.5 g Trypsin, 0.2 g EDTA, 0.85 g NaCl/L) to promote detachment. After incubation at room temperature (RT) for 5 mins, cells were split 1:3 into new T175 flasks and provided with 30 mL fresh media.

2.3.2. HEK293T cell culture

HEK293T cells (ATCC® #CRL-3216) were kindly provided by Andrew Lever (Department of Medicine, University of Cambridge). Cells were maintained in 75 cm^2 culture (T75) flasks in DMEM-10 and incubated at 37°C, 5 % CO_2. Confluent cultures were passed every 4-5 days by washing with PBS and adding 4 mL Trypsin-EDTA. After incubation at room temperature (RT) for 5 mins, cells were split 1:5 into new T175 flasks and provided with 20 mL fresh media.

2.3.3. Kasumi-3 cell culture

Kasumi-3 cells (ATCC® #CRL-2725) was maintained in 75 cm^2 culture (T75) flasks in RPMI-20 at a concentration between 3×10^5 - 3×10^6 cells/ml. Cultures were passed every 5 days by splitting 1:2 into new T75 flasks and provided with 20 mL fresh media.

2.3.4. THP-1 cell culture

THP-1 cell lines (ATCC® #TIB-202) stably expressing shRNA to human PML were kindly gifted by Thomas Stamminger (University Erlangen-Nurnberg, Erlangen, Germany). Cells were maintained in T75 flasks in RPMI-20 at a concentration between 3×10^5 - 3×10^6 cells/ml. Cultures were passed every 3 - 4 days by splitting 1 : 2 into new flasks and provided with 20 mL fresh media.

2.3.5. Freezing and long term storage

For long term storage, cells were pelleted by centrifugation and resuspended to a density of 1×10^6 cells per ml in freeze mix. The cell suspension was aliquoted into cryogenic vials, which were stored in a Mr. Frosty ™ Freezing Container (Nalgene) at -80°C overnight, before being transferred to a liquid nitrogen cryogenic storage system at -196°C for future use. Immortalised cell lines were resuscitated at regular intervals as a precaution against phenotypic changes associated with prolonged cell culture.

2.4. VIROLOGY

2.4.1. Virus propagation

Cultured HFFs were grown to 75 % - 80 % confluence in T175 flasks and infected at a low MOI (0.01 FFU/cell) in 6 mL DMEM-10 for 3 hours at room temperature with gentle rocking. Residual media was removed and replaced with 25 mL fresh DMEM-10, and flasks were then incubated at 37°C, 5 % CO_2. Upon 70 % infection or greater, as determined visually by microscopy, the media was harvested and replaced with 25 ml fresh media. Pooled media was centrifuged at 800 *g* for 20 mins to remove cellular debris, with the resulting supernatant stored at -80°C. This entire process was repeated every 2 to 3 days until all the cells had become detached from the flask, indicating non-viability. Accumulated frozen infectious media was thawed at 37°C and spun at 26000 *g* for 3 hours at room temperature using an Avanti J-25 Ultracentrifuge (Beckman-Coulter). Supernatant fractions were then discarded, and virus pellets resuspended in an appropriate volume of X-VIVO™ 15, aliquoted for seed and working stocks, and stored at -80°C.

2.4.2. Virus titre determination

HFFs were seeded 1 day prior to infection at a 2×10^4 cells/well density in 96-well flat bottom plates (Corning) and incubated at 37°C, 5% CO_2. On the day of infection, serial dilutions (1:2 - 1:2056) of working virus stocks were added to cells and incubated for 2 - 3 hours at room temperature with gentle rocking. Virus solutions were aspirated and replaced with fresh media, and cells were incubated overnight at 37°C, 5 % CO_2. Spent media was then aspirated off and cells were fixed in ice-cold ethanol (70%) for 3 hours at -20°C. Residual ethanol was removed by washing cells in PBS for 10 minutes. Cells were stained with 100 µL/well 1 µg/mL mouse anti-HCMV IE monoclonal antibody (MAB8141, Millipore) prepared in PBS and incubated at RT for 1 hour. Surplus antibodies were removed by washing cells in PBS for 10 mins. Cells were then stained with 2 µg/mL goat anti-mouse Alexa Fluor® 594 antibody (Life Technologies) and 1 µg/mL Hoechst 33342 (Sigma-Aldrich) in PBS for 1 hour at RT in the dark. After the cells were washed twice in PBS, staining was visualised under UV and green illumination, using a Eclipse TE300 UV microscope (Nikon) and a C5810-01 colour chilled 3CCD camera (Hamamatsu Photonics KK). The viral titre was expressed in terms of fluorescent focus units per mL (FFU/mL), which may be calculated as:

Viral titre = average no. of fluorescent foci per well × dilution factor × volume factor

2.4.3. Experimental virus infection

To achieve the required MOI for each infection, cells were enumerated accordingly and aliquots of stock virus were diluted in appropriate media.

Suspension cells (e.g. $CD34^+$ cells, Kasumi-3 and THP-1 cell lines) were cultured for at least 4 hours at 37°C, 5 % CO_2 prior to infection. Infections were carried out in suspension using 14 ml polypropylene tubes (BD Falcon). After pelleting by centrifugation at 300 g for 15 minutes, cells were resuspended in 350 µL virus-containing media for 3 hours at 37°C, 5 % CO_2, with gentle agitation every 30 mins. Cells were then diluted in fresh media, pelleted and resuspended before being plated.

Adherent cells (e.g. $CD14^+$ cells and terminally differentiated myeloid cells) were cultured for at least 24 hours in appropriate media at 37°C, 5 % CO_2 prior to infection. Media was removed from wells and cells washed with PBS. Virus-containing media was added to cells at 1.5 ml/well in 100 mm plates, 300 µL/well in 24-well plates and 50 µL in 96-well plates, and then incubated at room temperature for 3 hours with gentle rocking, before being aspirated and replaced with fresh media.

2.5. MYCOPLASMA TESTING

All cell lines were routinely tested for *Mycoplasma* contamination using MycoAlert® Mycoplasma Detection kit (Lonza). If positive results were obtained, cells would be treated every other day for 2 weeks with Plasmocin (InvivoGen) and then grown in antibiotic-free media for an additional two weeks before being confirmed mycoplasma negative.

2.6. CHROMATIN IMMUNOPRECIPITATION ASSAY

The following chromatin immunoprecipitation (ChIP) technique was modified from Bresnick *et al*. Cultured cells (2×10^7 per condition for $CD14^+$ monocytes; 1×10^6 per condition for $CD34^+$ cells) were incubated with formaldehyde at a final concentration of 1.0 % for 10 minutes at room temperature with gentle agitation to promote protein-DNA cross-linking. Glycine (0.125 mol/L) was subsequently added to quench the reaction. Cells were collected by scraping and centrifuging at 2,000 *g* for 5 minutes at 4°C and washed

twice in PBS. Nuclei were isolated by resuspending the pellet in ChIP cell lysis buffer for 10 minutes on ice, followed by centrifugation at 2,000 *g* for 5 minutes at 4°C. Nuclei were then washed twice in ice-cold ChIP wash buffer and centrifuged at 2,000 *g* for 5 minutes at 4°C and subsequently in ice-cold shearing buffer at 2,000 *g* for 5 minutes at 4°C, before being resuspended in 300 µL shearing buffer. Samples were sonicated using a Bioruptor® pico (Diagenode) for 9 repeated cycles of 30 seconds 'on' and 30 seconds 'off'. Soluble chromatin was precleared by addition of 200 µL Protein A-Sepharose (50 % suspension) containing 1% bovine serum albumin (BSA) and sonicated herring sperm (HS) DNA at 200 µg/m, and incubated at 4°C for 1 hour with gentle rotation. An aliquot of precleared chromatin was removed (input) and used in the subsequent polymerase chain reaction (PCR) analysis. The remainder of the chromatin was diluted with IP dilution buffer to a final volume of 800 µL and incubated with 5 µg of the appropriate antibody or equivalent control overnight at 4°C with gentle rotation. Immune complexes were collected by addition of 60 µL of Protein A-Sepharose for 2 hours at 4°C with gentle rotation. Protein A-Sepharose pellets were washed twice with 500 µL aliquots of IP wash buffer 1, once with IP wash buffer 2, and twice with TE buffer. Immune complexes were eluted twice with 150 µL of IP elution buffer. RNAase A (0.03 mg/ml) and NaCl (0.3 mol/L) were added, and crosslinks were reversed by incubation at 65°C for 5 hours to overnight. Eluted DNA was then digested with Proteinase K (0.24 mg/mL) for 2 hours at 45°C, before being purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and collected in 30 µL DNAse-free water. Aliquots of ~2 µL were analysed by quantitative PCR (qPCR) using the appropriate primer pairs.

2.7. PCR-BASED ANALYSIS

2.7.1. Quantitative reverse-transcription PCR

One-step quantitative reverse-transcription PCR (qRT-PCR) was performed to determine levels of viral IE and UL138 gene expression. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalise the data for each experiment. All reactions were performed with water-only and RT-negative controls.

For isolation of total RNA, cell cultures were directly harvested in in TRIzol® reagent (Invitrogen). RNA was then purified from TRIzol®-lysed samples using the RNEasy Mini Kit (Qiagen) according to the manufacturer's instructions, eluted in RNAse-free water, and

quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Samples were subsequently stored at -80°C.

For SYBR Green qRT-PCR, the following primers and probes were used: IE, 5'-CGG GAC AGG AAG ACA TCA AGC CCG and 5'-TTG TTG CGG TAC TGG ATG GTA AA, UL138, 5'-CAT GGC TAC GGT GGT GAA CTG and 5'-CGG GCG TCG ATC TGT TGA AAC CCG; GAPDH, the same set as above. Reactions were performed using QuantiTect SYBR® Green RT-qPCR kit (Qiagen) according to the manufacturer's instructions and the samples amplified and detected using an ABI 7500 Fast Real Time PCR machine (95°C for 15 s and 60°C for 45 s).

Quantification of RNA was performed using the 'comparative Ct method' ($2^{-\Delta\Delta Ct}$). Absence of a detected signal indicated that products failed to cross the threshold cycle after the maximum number of cycles (60) was performed.

To evaluate amplification specificity, melt curve analysis was performed immediately after amplification by measuring the reduction in the fluorescence during a linear temperature transition from 60°C to 95°C at a rate of 0.5°C/sec

2.7.2. Quantitative PCR

Quantitative PCR (QPCR) was performed to determine levels of enrichment of epigenetic histone marks for various cellular and viral targets. Lyophilised desalted primers in table below were purchased from Sigma and reconstituted in DNAase-free water at 1 µg/ml and stored at -20°C. Experiments were performed using QuantiTect SYBR® Green RT-qPCR kit according to the manufacturer's instructions and the samples amplified and detected using an ABI 7500 Fast Real Time PCR machine (95°C for 15 s and 60°C for 45 s).

Name	**Forward primer (5' - 3')**	**Reverse primer (5' - 3')**
Sequence of oligos used for qPCR		
LUNA promoter	GCGGGTTCCAATCAGCAGCAGC	CAGCTACCTTGGCACCTCCGG
UL144 promoter	TCCATGGGAATCAACGGATC	TCCGAACTTTTATACACGCC
UL138 promoter	CGGGGTACCCGGCGTAAGAGAAAC CGA	CCGCTCGAGGCCAACTGTCCTGGTG GT
MIEP	TGGGACTTTCCTACTTGG	CCA GGC GAT CTG ACG GTT
TLR4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC
ZNF180	TGATGCACAATAAGTCGAGC	TGCAGTCAATGTGGGAAGTC

2.8. LUCIFERASE REPORTER ASSAY

2.8.1. Transfection of Kasumi-3 and THP-1 cell lines

Kasumi-3 and THP-1 cell lines were plated at 1 - 2 × 10^6 cells/well in 6-well culture plates and incubated overnight at 37°C, 5 % CO_2 prior to transfection. Cells were co-transfected with an effector expression vector (either LUNA, GATA-2 or both) along with a luciferase reporter plasmid (either pGL3-UL144 or pGL3-LUNA) in a 2 : 1 / effector : reporter or 1 : 1 : 1 / effector : effector : reporter ratio, each totalling 1.50 µg DNA. Transfections were performed with the V-001 program using a Nucleofector™ 2b device (Amaxa) in conjunction with Nucleofector® Kit R (Amaxa) according to the manufacturer's instructions and cells were incubated for 48 hours at 37°C, 5 % CO_2.

2.8.2. Luciferase assay

Cellular lysates for assaying were prepared using the luciferase assay system (Promega) according to the manufacturer's instructions. Briefly, cells were washed in PBS and then resuspended in cell culture lysis reagent. 20 µL lysate from each condition was then plated into 96-well luminescence plates (Greiner) in duplicate format. Luciferase expression activity was subsequently measured using a GloMax®-Multi Microplate Multimode reader (Amaxa), in which 100 µL luciferin substrate was delivered to each well and the luminescence produced measured after a 1 second delay with an integration time of 10 seconds.

2.9. CO-IMMUNOPRECIPITATION ASSAY

2.9.1. Transfection of HEK293T cell line

HEK293T cells were seeded into 100mm dishes and cultured for at least 4 hours at 37°C, 5 % CO_2 prior to transfection. Once cells had reached ~70 % confluency, they were co-transfected with LUNA and GATA-2 effector expression vectors in a 1:1 ratio, totalling 1.50µg DNA. Transfections were performed using TransIT®-293 transfection reagent (Mirus Bio) according to the manufacturer's instructions, and cells were then incubated for 48 hours at 37°C, 5 % CO_2.

2.9.2. Co-immunoprecipitation

The following procedure for co-immunoprecipitation (co-IP) experiments was adopted from ImmunoCruz™ IP/WB Optima E system (Santa Cruz Biotechnology, Inc.) with modifications. Briefly, whole cell lysate was prepared by washing cells twice in PBS, followed addition of 1mL ice-cold RIPA buffer with repeated aspiration for 10 minutes. Cellular debris was removed by centrifuging at 10000 *g* at 4°C for 15 minutes, and transferring the supernatant to new 1.5 ml Eppendorf tubes. Antibody agarose conjugates were formed by incubating 5µL rabbit polyclonal anti-FLAG® antibody (F7425; Sigma) with 50 uL suspended (25 % v/v) IP matrix in 500 µL PBS. 200 µL of clarified lysate with then incubated with the above complexes overnight at 4°C with gentle rotation. IPs were then washed three times in 750 µL lysis buffer for 20 minutes at 4°C before agarose beads were incubated in 75 µl of 2 × SDS-PAGE sample buffer containing 5 % β-mercaptoethanol at 85°C for 10 min to elute proteins.

2.9.3. Western blotting

Samples were boiled at 95°C for 2 mins before being loaded in equal amounts (20 µL) on a 12 % bis-acrylamide Resolving gel overlaid with a 12 % Stacking gel. Proteins were separated by electrophoresis using SDS-PAGE, before being electroblotted on to Hybond nitrocellulose membranes (Amersham) using Transfer buffer. Samples were than pre-treated in blocking solution (5 % milk in 1 × PBS) at room temperature for 1 hour, then incubated with primary rabbit anti-GATA-2 antibody (Cell Signalling Technologies) diluted 1 : 1000 in blocking solution overnight at 4°C. Primary antibodies were detected using HRP-conjugated rabbit anti-mouse IgG antiserum (Santa Cruz) diluted 1 : 2000 in blocking solution at room temperature for 1 hour. All antibody incubations were followed by 3 washes using 0.1 % Tween-20 in PBS. Detection of proteins was carried out using Chemiluminescence (ECL, Amersham) according to the manufacturer's instructions, followed by autoradiographical exposure to X-ray films (Super FX, Fujifilm).

2.10. INDIRECT IMMUNOFLUORESCENCE

2.10.1. Specimen preparation

Kasumi-3 (stably transfected) and THP-1 cells were suspended in solution and measured by trypan blue dye exclusion using a haemocytometer. Approximately 3 - 4 × 10^4 cells in 200 µL PBS were cytospinned onto polylysine-coated glass slides at 500 rpm for 5 min. Each slide was then fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature, before being washed and maintained in PBS. Monocytes were washed in PBS and then fixed in 4 % paraformaldehyde for 10 minutes at room temperature, before being washed and maintained in PBS.

2.10.2. Staining procedure

Fixed cells were permeabilised in 0.1 % Triton-X-100 for 2 minutes before being washed in PBS. Cells were subsequently incubated with mouse monoclonal anti-FLAG M2 (1 : 500; Sigma) and goat anti-PML (1:500, clone N19; Santa Cruz Biotechnology) for 1 hour. After washing in PBS, slides were incubated with donkey anti-goat Alexafluor 488 nm and rabbit anti-mouse Alexafluor 594 nm antibodies (1:1000; Merck Millipore) for 1 hour and then visualised as described above.

2.11. GENOME FLUORESECENT *IN SITU* HYBRIDISATION

Genome fluorescent in situ hybridisation (FiSH) experiments were performed by Matthew Reeves using a previously published protocol[298]. Infected $CD34^+$ cells were infected and cultured on glass coverslips for 7 days prior to processing. Briefly, cells were fixed in 4 % PFA, and following washing in PBS, were incubated in hybridization buffer (50 % formaldehyde / 10% dextran sulphate in PBS) for 1 hour at 37°C. Cells were then incubated with a fluorescently labelled HCMV cosmid DNA probe (Cy3-dCTP) spanning the major immediate early region for 90 seconds at 95°C in hybridization buffer followed by an overnight incubation at 37°C. Two washing steps in 2 × saline sodium citrate buffer (each 5 mins; at 60°C) were followed by a wash in PBS at room temperature and then stained, as described above, to detect PML localisation. Cells were then visualised by confocal microscopy.

2.12. DESUMOYLATION / "SUMO-CHOP" ASSAY

A SUMO-CHOP assay kit (Lifesensors, Malvern, PA) was used to identify isopeptidase activity. Briefly, following cleavage of a SUMO3-reporter system by a candidate isopeptidase activity, the reporter becomes free to act upon its substrate, thereby generating a fluorescent signal which can be measured to quantify the level of isopeptidase activity. Recombinant LUNA was generated from BL21 bacteria transfected with either PET102UL82as or pCMV-Tag2b-FLAG-g233c using previously published methods[263]. Provided aliquots of SENP2core were used as the control isopeptidase.

2.13. STATISTICS

Unless otherwise stated, a comparison of the means were performed using unpaired, two-tailed Student's *t* test with p values reported or stated as being not significant (NS). Where multiple comparisons were made, one-way ANOVA tests were performed followed by Tukey HSD post-test analysis in order to determine statistical significance.

3. Modulation of cellular ND10 during latent HCMV infection

3.1. INTRODUCTION

HCMV is the prototypical member of the β-herpesvirus subfamily. Like all herpesviruses, HCMV establishes latency as a hallmark of infection, which is integral to promoting lifelong persistence as well as becoming a widespread human pathogen. While most acute infections with HCMV are asymptomatic in healthy individuals, they are otherwise associated with opportunistic disease in those with immature or compromised immune systems, such as neonates or recipients of organ transplantation, respectively[299,300]. Therefore, in addition to primary infection, latent virus and their ability to reactivate represent a serious clinical threat to vulnerable patient populations, underscoring the importance of understanding the mechanisms that govern this major life cycle process.

Cells of the early myeloid lineage, such as $CD34^+$ progenitors and $CD14^+$ monocytes, represent major sites of HCMV latency *in vivo*[209]. During latency, the virus genome is maintained extrachromosomally as a circular episome in the concomitant absence of lytic gene expression. Importantly, the expression of major IE (MIE) genes IE1 and IE2, which are the main viral transactivators responsible for initiating the lytic cascade of virus gene expression, is suppressed. This is maintained by specific post-translational histone modifications around the MIE promoter (MIEP), amounting to the formation of repressed chromatin[170]. In latently infected $CD34^+$ cells, the MIEP has been found to be associated with heterochromatin protein-1 (HP-1), along with the presence of repressive histone marks and absence of histone acetylation, thus reflecting a closed chromatin configuration[219]. Indeed, this configuration has also been similarly observed in latently infected $CD14^+$ monocytes[167]. However, upon differentiation of these cells to a mature phenotype, associated changes in the nuclear environment results in activation of the viral MIEP and the reactivation of productive replication.

The silencing of HCMV DNA through chromatinisation and epigenetic histone modifications are not only observed during latency, but also feature during lytic infection of permissive cells as part of an intrinsic immune response[181]. During infection with a number of nuclear-

replicating DNA viruses, including HCMV as well as other herpesviruses, discrete subnuclear structures, termed ND10, rapidly become localised to sites of deposited viral genomes[301]. These structures resemble dynamic clusters of protein found within the nucleoplasm, but are primarily defined by the presence of core constituent proteins PML, hDaxx and Sp100[177]. Several studies have demonstrated that these major ND10 components independently contribute to the silencing of herpesvirus gene expression, giving rise to the notion that ND10 serve to restrict viral replication by generating a transcriptionally repressive environment around incoming viral DNA[178,186,197,302]. Notably, hDaxx is implicated in generating a repressed chromatin structure around the HCMV MIEP at very early times post-infection, thereby inhibiting IE gene expression[185]. Yet, unsurprisingly, most, if not all herpesviruses, encode discrete factors to overcome the cellular restriction imposed by ND10, which often leads to ND10 becoming disrupted as infection proceeds. Thus, with respect to HCMV, the tegument-derived pp71 protein facilitates the proteasomal degradation of hDaxx, whereas the IE1 protein induces the complete dispersal of ND10 by selectively modifying PML, the main scaffold protein for ND10 assembly[185,197,198,303]. In both cases, the respective function of each viral factor correlates with efficient IE gene expression and subsequent productive replication.

While it is clear that lytic HCMV infection results in the potent disruption of cellular ND10, little is known about what happens to ND10 in the context of latency. Presently, it is understood that viral pp71 is sequestered in the cytoplasm of latently infected cells, which thus prevents it from relieving the transcriptional repression of IE genes mediated by ND10-associated protein hDaxx[304]. Since the MIEP is known to adopt a repressed chromatin conformation during latent infection, as it also appears to do during the 'pre-IE' stages of lytic infection, it has been suggested that the inability to antagonise the repressive functions of ND10 during latent HCMV infection may aid in their general capacity to form silenced chromatin over latent viral genomes, and in so doing, contribute to latency establishment[305,306]. Therefore, the primary aim of this investigation was to determine what effect HCMV had on ND10 structures in the latently infected cell, using undifferentiated myeloid cells as models of experimental latency.

3.2. RESULTS

3.2.1. ND10 structures are disrupted during latent HCMV infection

Cellular ND10 structures are represented by discrete foci appearing within the interchromosomal space of the nucleus. They are found in virtually all cell types, with their diameter ranging from 0.2 to 1 μm and frequency varying from a minimum of between three to ten, up to a maximum of 15 to 30, depending on cell type and status[37,307,308]. Discerning microscopically between individual ND10 foci is best achieved through examination with a very high magnification lens, which usually necessitates the use of glass slides that provide minimal refraction of light. Unfortunately, cells used throughout project were often difficult to mount successfully on to coated and uncoated glass slides, and as a result, plastic had to be used instead. Because plastic is incompatible with the effective use of very high magnification objectives, I chose to define cells as having non-disrupted (intact) ND10 if a minimum of five discrete foci were present and disrupted ND10 if under five.

The PML protein is a major constituent component of ND10 that plays an important role in their assembly and maintenance. Since PML is critical for the structural integrity of ND10, loss of PML consequently leads to a dispersal of ND10 foci[309]. This is well established for a number of cell lines used to model lytic infection of HCMV, but is also clearly the case for myeloid cells, which are known to routinely establish latent HCMV infection[220]. To this end, shRNA-mediated PML-knockdown of myelomonocytic THP-1 cells show clear disruption of ND10 (Figure 3.1).

Early myeloid progenitors constitute sites of latent HCMV carriage *in vivo*[238]. Previous reports support their use as models of experimental latency, in which following infection, there is an absence of IE gene expression that correlates with a lack of infectious viral spread[237]. To evaluate the disruption of ND10 bodies during latency, primary $CD34^+$ stem cells were also infected with the HCMV reference strain Merlin. Similarly, primary $CD14^+$ monocytes were also infected with GFP-expressing virus of the low-passage strain TB40-BAC4. Here, an opportunity should be taken to note that any analysis of global changes in HCMV-infected cells of the myeloid lineage is marred by the low frequency at which the virus appears to infect these cells *in vitro* unless very high MOI are used[310]. However, previous analysis of $CD34^+$ cells carrying HCMV genome following experimental latent infection with low-passage HCMV isolates at MOI = 5 (based on fibroblast infection) have shown that up to 60-70 % of cells carry the HCMV genome by *in situ* hybridisation assays (S. R. McGregor

Dallas & J. Sinclair, unpublished observations)[311]. Hence, I reasoned that this level of experimental latent infection should still result in global phenotypic changes that would be discernible. FISH analysis, performed using a fluorescent Cy3-labeled HCMV cosmid DNA probe, showed that virally-infected $CD34^+$ cells were devoid of ND10 compared to uninfected bystanders, consistent with both a demonstrable lack of PML staining and cellular enumeration (Figure 3.2). Likewise, using immunofluorescence microscopy, the same ND10 disrupted phenotype was observed in latently infected $CD14^+$ monocytes relative to uninfected bystanders (Figures 3.3).

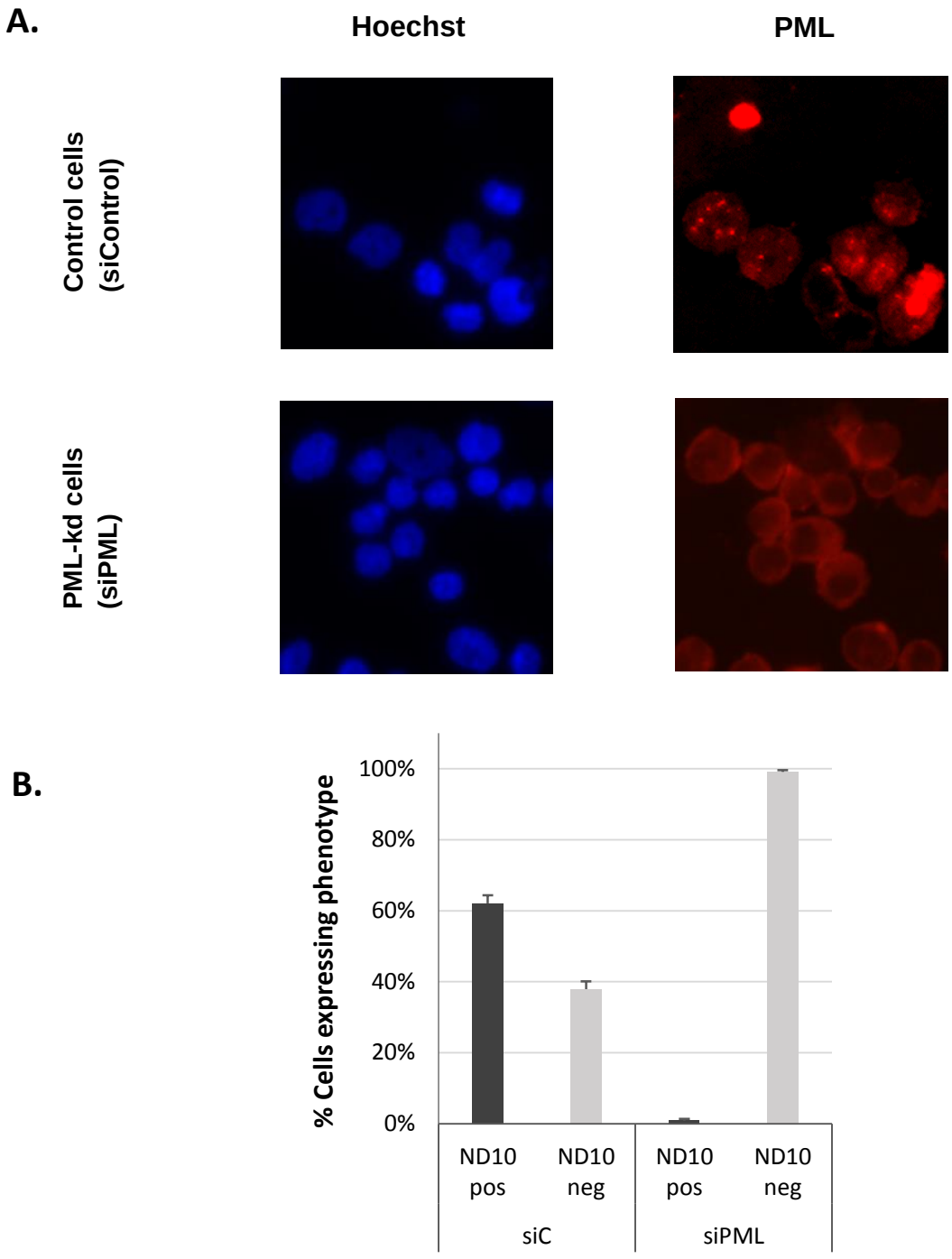


Figure 3.1. Depletion of PML prevents formation of ND10 structures in myeloid cells

(A) THP-1 cells stably expressing shRNA to PML (siPML) or inactive shRNA (siC) were fixed and stained for PML, then analysed by immunofluorescence microscopy (magnification; × 100). (B) The number of ND10 positive or negative cells were enumerated using ImageJ software. Bars represent averages of 5 fields of view of 100 cells, each showing standard deviation.

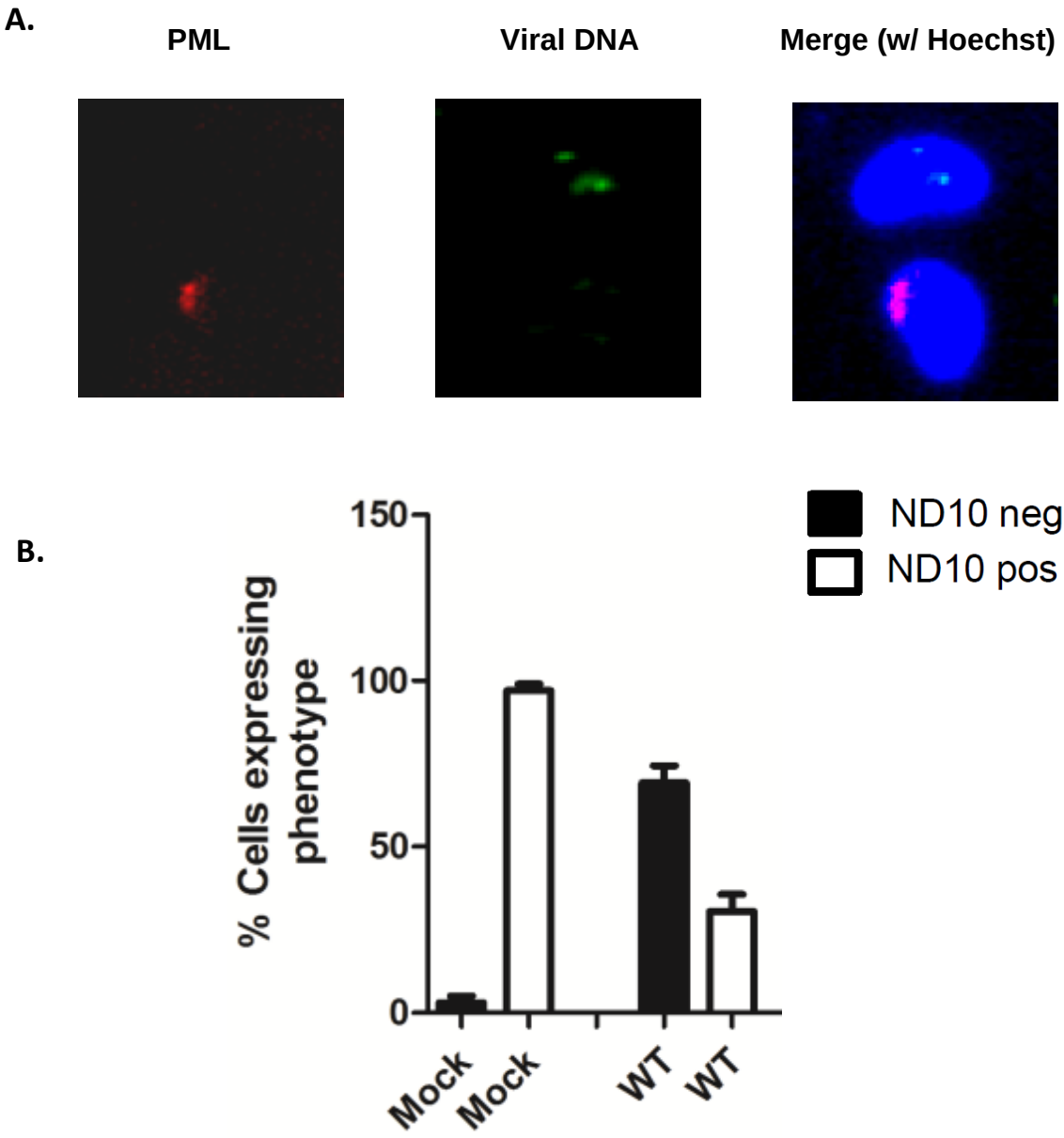
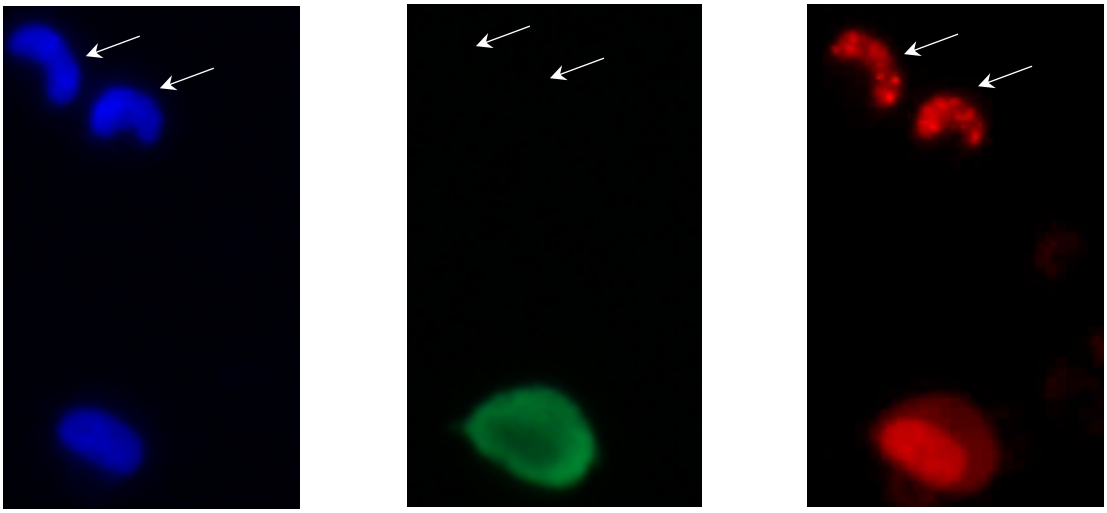


Figure 3.2. HCMV-infected CD34$^+$ myeloid cells show disrupted ND10

Primary CD34$^+$ cells were infected with wild type Merlin at MOI = 5 for 7 days to promote latency establishment. (A) Infected cells were identified by genome FISH (red) and subsequently stained for PML (green) as an indicator of ND10 integrity (magnification; × 100). (B) The number of infected ND10 positive or negative CD34$^+$ cells were scored from 10 fields of view at 7 days post infection with wild type Merlin (WT).



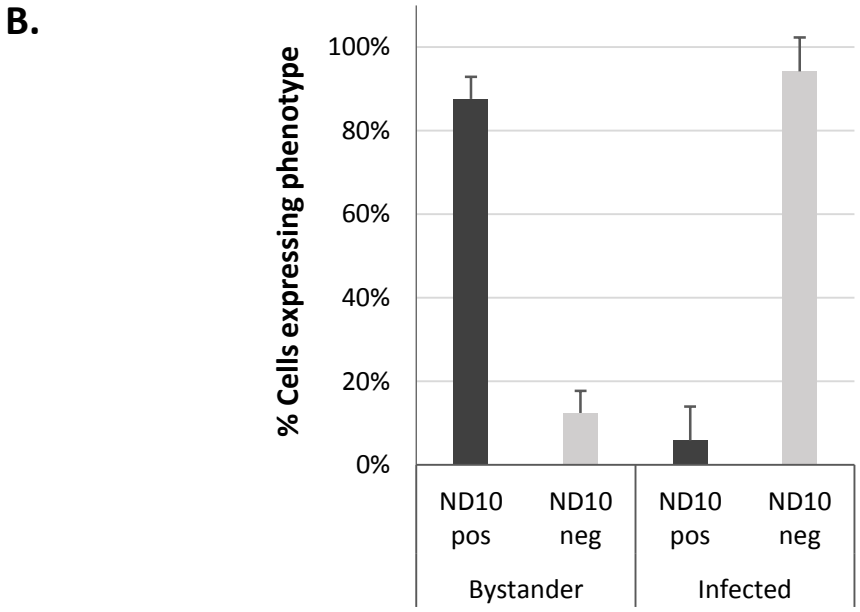
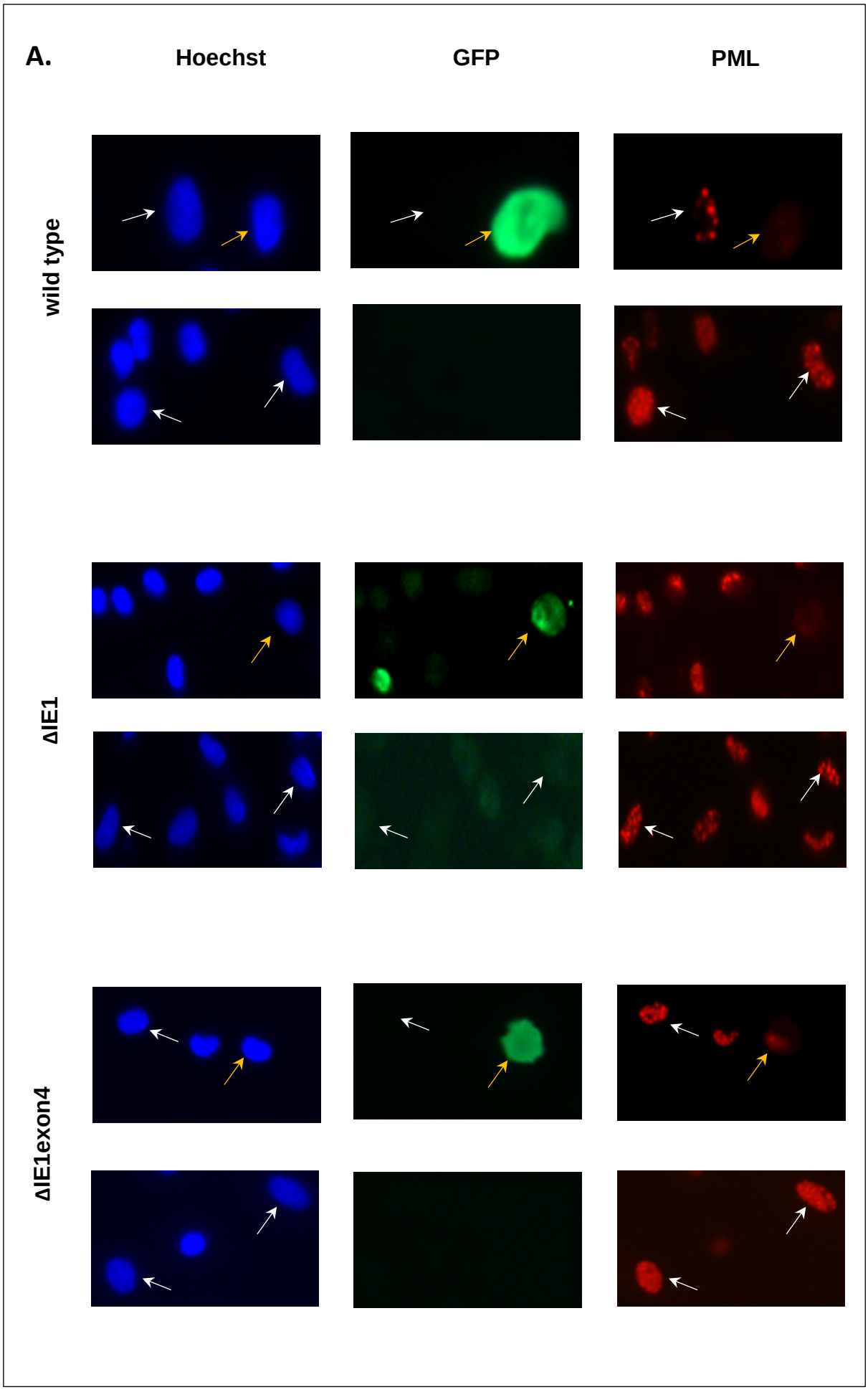


Figure 3.3. HCMV-infected CD14^{+} monocytes show disrupted ND10.

Primary CD14^{+} monocytes were infected with wild type SV40-GFP-TB40-BAC4 at MOI = 5 for 3 days to promote latency establishment. (A) Cells were stained using anti-GFP antibody for GFP-tagged strain TB40-BAC4 (green) and PML (red); hoechst DNA staining (blue) indicates position of nuclei (magnification; ×100). *Yellow arrows* point to a successfully infected cell exhibiting disruption of ND10 by PML staining. *White arrows* point to uninfected cells containing intact ND10. (B) The relative number of latently infected (GFP+) or uninfected bystander cells (GFP-) in the population coincident with intact ND10 were enumerated using ImageJ software. Bars represent averages of 5 fields of view of 100 cells, each showing standard deviation.

3.2.1. Deletion of the IE1 coding region does not prevent ND10 disruption in latently infected cells

HCMV proteins pp71 and IE1 have been identified as viral factors responsible for overcoming the negative regulatory effects of ND10 during lytic infection. One contributing factor to latent infection has been posited to be the failure to deliver viral pp71 to the nucleus in non-permissive cell types[304]. However, by contrast, the expression of IE1 has been detected in models of experimental latency, albeit only transiently and weakly[248]. Notably, it has been observed that the IE1 exon 4 mRNA is transcribed during latent infection of haematopoietic progenitor cells, resulting in the latent expression of a distinct IE1 protein species[295]. Previous research has shown that the exon-4 segment of the IE1 coding sequence is important in conferring the ability of IE1 to induce loss of SUMOylated PML that leads to ND10 disruption[198]. Consequently, despite its classification as a lytic gene product critical for productive infection, it can be argued that IE1 may play a role in mediating ND10 disruption during latency. To evaluate this possibility, primary $CD14^+$ monocytes were infected using IE1 mutant TB40-BAC4 viruses, which were then assayed for ND10 disruption. Consistent with previous results, deletion of IE1 or of its corresponding exon-4 segment did not prevent disruption of ND10 in the context of latent infection (Figure 3.4).


A.
Hoechst
GFP
PML
wild type
ΔIE1
ΔIE1exon4

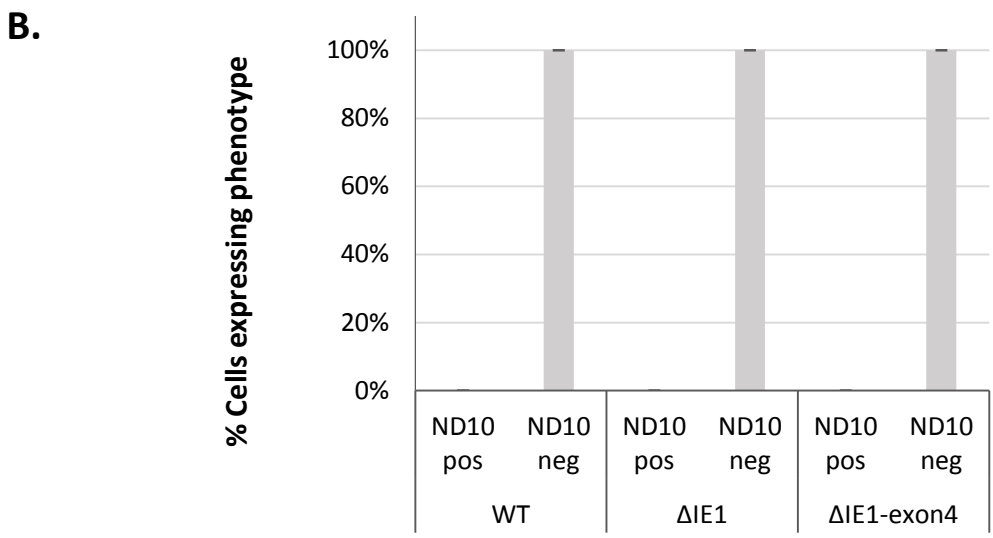


Figure 3.4. IE1 does not prevent disruption of ND10 in context of latent infection

$CD14^{+}$ monocytes were infected with eGFP-expressing TB40-BAC4 wild type, IE1 deletion (ΔIE1), IE1 exon-4 deletion (ΔIE1exon4) viruses at MOI = 5 for 3 days to promote latency establishment. (A) Cells were stained for GFP (green), PML (red) or nuclei (blue) (magnification; × 100). *Yellow arrows* point to a successfully infected cell exhibiting disruption of ND10 by PML staining. *White arrows* point to uninfected cells containing intact ND10. (B) The number of ND10 positive or negative cells were enumerated using ImageJ software. Bars represent averages of 5 fields of view of 100 cells, each showing standard deviation.

3.2.2. Direct expression of LUNA promotes disruption of ND10 in undifferentiated myeloid cells

Research conducted by our laboratory has sought to address the functions of a number of HCMV latency-associated gene products during latent infection. The HCMV LUNA protein is an example of one such latent viral gene product whose direct effects on the latently infected cell remain unknown, but is otherwise understood to be important for efficient latent carriage *in vitro*[266].

While investigating the role of LUNA under conditions of viral reactivation and lytic infection, Emma Poole in collaboration with Matthew Reeves (University College London, UK), observed that the isolated expression of LUNA in fibroblasts – which are permissive for lytic infection – caused them to become devoid of ND10 (Poole *et al*. under review). Thus, LUNA, like HCMV lytic IE1, may be involved in disrupting ND10 structures. Given its latency-associated expression, this means LUNA could be the factor responsible for promoting the disruption of ND10 in undifferentiated myeloid cells. Hence, to assess the potential role of LUNA in mediating ND10 disruption under conditions of latency, transient transfection assays were performed to express the protein in isolation and monitor its effects accordingly. Kasumi-3, a $CD34^+$ undifferentiated leukaemia cell line, was used as a tractable model for this study as not only was it transfectable, but it had also been shown in previous studies to support latent infection with clinical isolates of HCMV[312].

Direct expression of LUNA in Kasumi-3 cells strongly coincided with a lack of distinct PML foci, arguing for the existence of its role in disrupting ND10 (Figure 3.5). Moreover, the images indicate a predominantly nuclear localisation of the LUNA protein. However, this is not consistent with previously published data that shows LUNA localising to the periphery of the nucleus in latently infected $CD14^+$ monocytes one day post-infection[266]. To that end, it is possible that the cellular localisation of LUNA may be influenced by different cell types of haematopoietic origin, but also the context by which it is expressed, either directly or through latent viral infection.

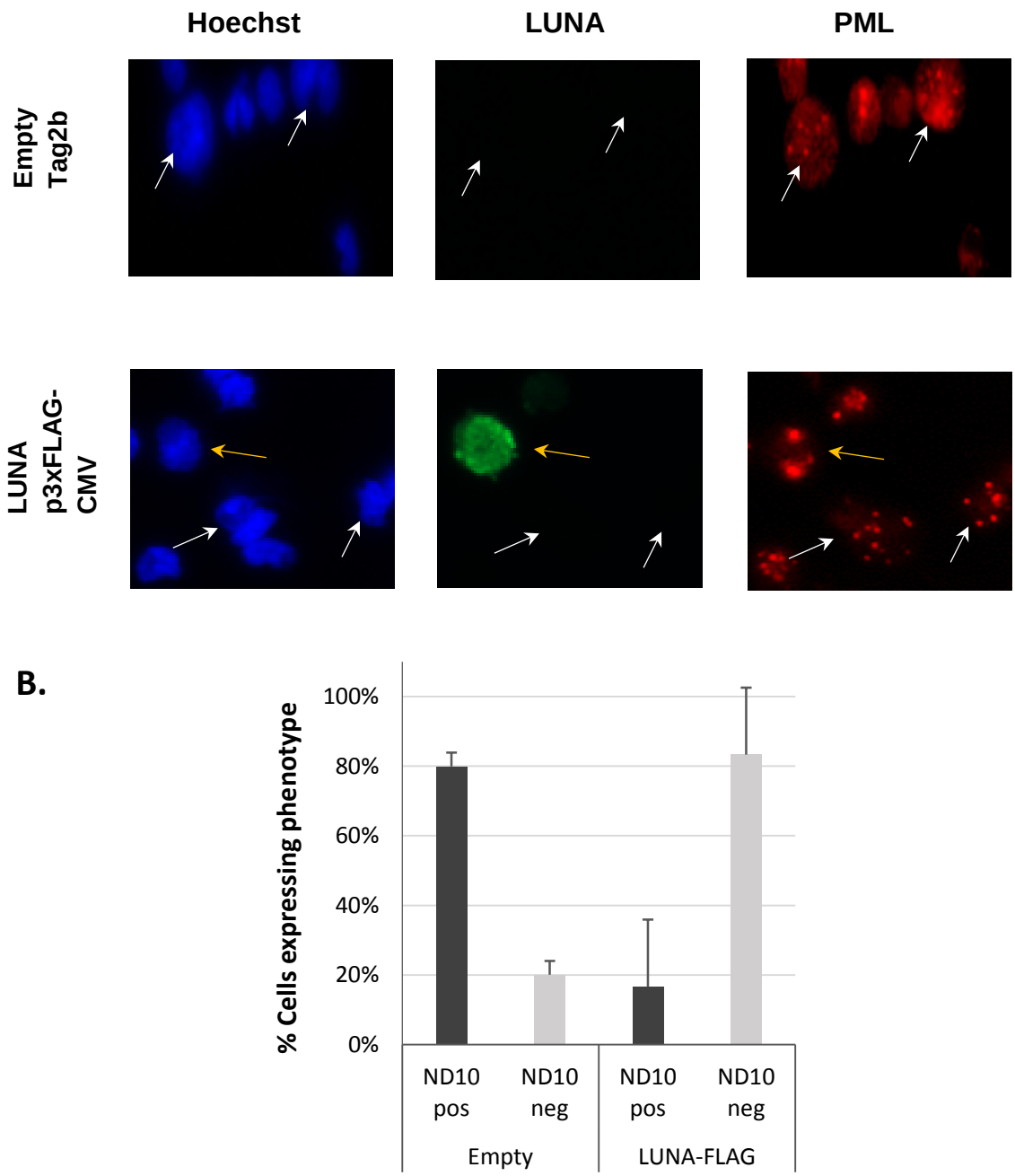


Figure 3.5. Myeloid cells transfected to express LUNA exhibit disrupted ND10

The early myeloid cell line, Kasumi-3, was transiently transfected with expression plasmids encoding wild type FLAG-LUNA fusion protein. (A) 48 hours post transfection, cells were cytospinned and stained for LUNA (green), PML (red) or nuclei (blue) (magnification; × 100). *Yellow arrows* point to a successfully infected cell exhibiting disruption of ND10 by PML staining. *White arrows* point to uninfected cells containing intact ND10. (B) The number of transfected ND10 positive or negative cells were enumerated using ImageJ software. Bars represent averages of 5 fields of view of 100 cells, each showing standard deviation.

3.3. DISCUSSION

Taken together, these results demonstrate that ND10 are disrupted during HCMV latency. Nuclear PML foci resembling intact ND10 are absent in early myeloid cells infected with wild-type phenotype viruses (Figures 3.2 and 3.3), reflecting similar observations made in PML knockdown cells (Figure 3.1). Additionally, the finding that IE1-null viruses failed to prevent the same effect from occurring excludes the possibility of this factor being responsible for mediating ND10 disruption in the latently infected cell (Figure 3.4). However, evidence points to a potential role for the viral LUNA protein in mediating disruption of ND10, notably, under conditions that support latency establishment *in vitro* where it is otherwise known to be expressed (Figure 3.5).

Given that the disruption of ND10 are essential for robust HCMV lytic gene expression and productive replication, the apparent loss of these structures during HCMV latency is striking. Indeed, it does not appear that ND10 are required for the maintenance of HCMV latency. Although ND10 have previously been suggested to contribute towards latency establishment by facilitating the silencing of incoming viral genomes, it is more likely that early myeloid cells inherently unable to support MIEP activity due to the differentiation status of the cell and the relative absence of positive effectors of the MIEP. Therefore, MIEP suppression during latency involves a plethora of cellular factors beyond those described thus far in the context of ND10-mediated suppression[102]. Whichever is the case, major components of ND10, such as PML, hDaxx and Sp100, appear unlikely, in themselves, to be the main cause of MIEP suppression during latency in view of recent evidence demonstrating that depletion of each of these proteins does not lead to HCMV IE gene expression in latently infected myeloid cell[302].

To strengthen the findings made above, further evidence regarding the disruption of ND10 should be brought to bear. Though subsequent investigations detailed throughout this report make use of LUNA mutant viruses, the current data does not indicate whether the disruption of ND10 in the context of latent infection is facilitated by events occurring downstream of PML gene expression. This can be addressed by appending the above analyses with western blotting of PML protein. One would predict that levels of PML should remain similar to uninfected controls during latent infection, but should otherwise be completely absent during shRNA-mediated knockdown of PML.

4. Functional analysis of LUNA during latent HCMV infection

4.1. INTRODUCTION

Cellular ND10 structures have increasingly been shown to act as an intrinsic line of defence that restricts lytic HCMV infection by targeting incoming viral DNA for transcriptional repression[37]. However, this antiviral response is rapidly counteracted by a number of virally-encoded proteins, resulting in either the degradation or relocalisation of ND10 components[181]. Indeed, their roles in disrupting the repressive functions of ND10 have been shown to be important for enabling efficient viral gene expression and productive viral replication in permissive cell types. Nevertheless, as demonstrated earlier, latently infected cells are also similarly devoid of ND10, which appears to be linked to the expression of the latency-associated viral LUNA protein. Because this particular finding argues for a relief of ND10-mediated repression, it is thus possible that LUNA might be able to affect virus gene expression during HCMV latency. Consequently, the aim of this investigation was to examine the effect of LUNA on levels of viral gene expression in latently infected cells.

4.2. RESULTS

4.2.1. Viral growth kinetics of recombinant Merlin strain mutants show no significant differences

To evaluate the function of LUNA during latent infection, a LUNA translation mutant was created through BAC recombineering of the Merlin backbone. Notably, although a stop codon was introduced to prevent LUNA expression, this did not disrupt the UL82 gene on the complementary DNA strand.

As a preliminary investigation, the growth of the LUNA-deficient virus was assayed for normal productive replication under lytic conditions in order to eliminate the possibility of any resulting phenotype observed during latency being linked to a defect in lytic replication. Consistent with previously published data, the deletion of LUNA did not majorly impact on

the growth of the virus in human fibroblast culture, which is otherwise permissive for lytic HCMV infection (Figure 4.1).

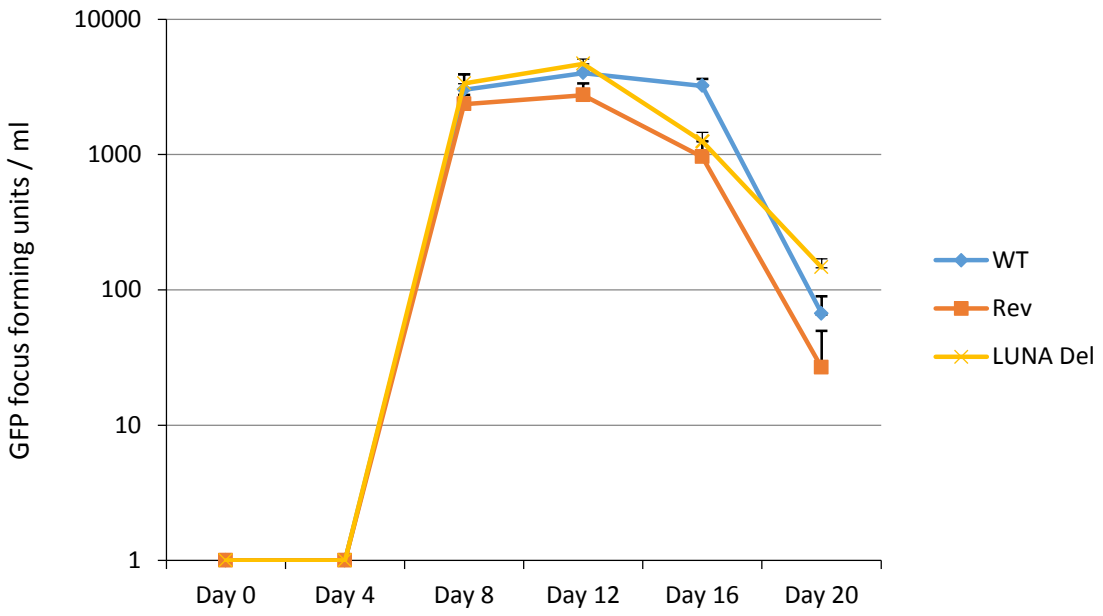


Figure 4.1. LUNA deficient viruses showed no significant defects in lytic viral growth

Wild type (WT), LUNA deletion or revertant viruses were generated by BAC recombineering using the Merlin backbone, which expresses a GFP-UL32 fusion protein. HFFs were plated at equal numbers and were subsequently infected at an MOI of 0.01. Viral growth was then measured over 10 days. Supernatants were titred for infectious virus production every 2 days by overlaying onto a monolayer of fibroblasts and monitoring for GFP expression by immunofluorescence microscopy.

4.2.2. LUNA is required for efficient viral gene expression during latent infection of primary CD34$^+$ cells

To address the impact of LUNA on virus gene expression during HCMV latency, RNA analyses were performed on undifferentiated CD34$^+$ cells infected with LUNA-deficient virus. Specifically, levels of viral UL138 and IE1 gene expression were assayed by RT-qPCR using total RNA as a target. UL138 is a latency-associated gene product, which has been shown to be important for establishing latency[313]. Recently published data suggest that UL138 directs repression of IE gene expression by preventing the removal of histone methylation marks at the MIEP[285]. By contrast, IE1 is a well-established MIE gene product, which is responsible for inducing the lytic cascade of virus gene expression[143]. Although, the latency-associated transcription programme is now thought to be far more complex than first thought, it is universally accepted that IE1 is poorly expressed in all natural and experimental models of latency, such that measurement of this transcript, in conjunction with UL138, serves as a useful indicator of latency establishment[254,314].

In general, analysis of latently infected myeloid cells characteristically gives levels of UL138 RNA higher than IE1 RNA (i.e. UL138/IE1 ratio is much greater than 1). In contrast, during lytic infection IE1 RNA level are substantially higher than UL138 level[215,315]. Consistent with this, CD34$^+$ cells infected with WT Merlin showed levels of UL138/IE1 RNA entirely consistent with a latent infection (Figure 4.2A). Moreover, an approximate four-fold and ten-fold decrease, respectively, in the levels of both UL138 and IE expression, were observed during latent infection with LUNA deletion virus compared to wild type viruses (Figure 4.2B). This finding is consistent with the view that LUNA is involved in regulating viral latency-associated gene expression. However, it also suggests that residual IE1 expression during latency is also decreased in a LUNA deleted virus.

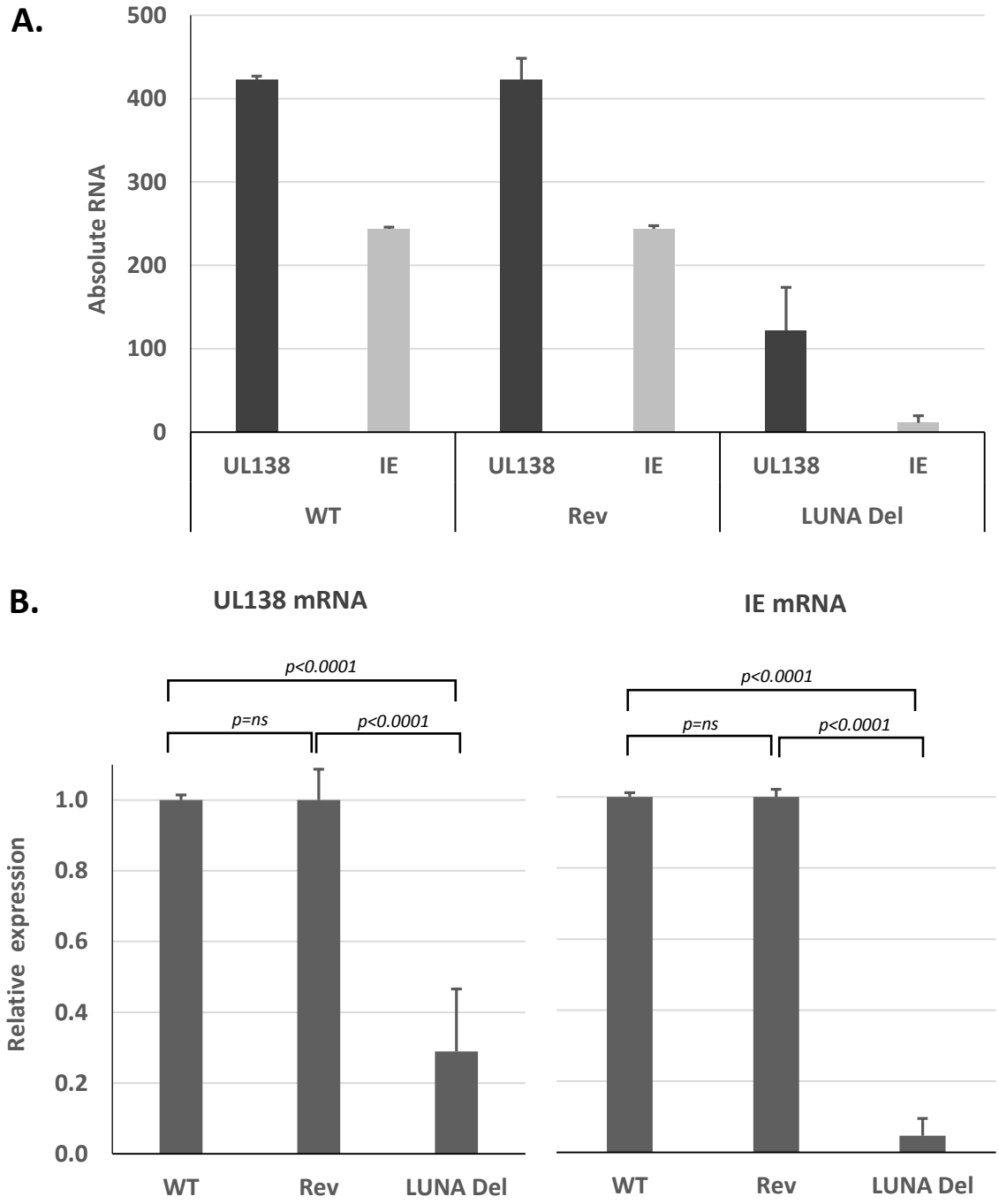


Figure 4.2. LUNA is required for efficient viral gene expression in latently infected CD34$^+$ cells

(A) RNA isolated from 1×10^5 CD34$^+$ stem cells latently infected at MOI = 5 with wild type (WT), revertant (Rev) and LUNA deletion (LUNA Del) at 7 days post infection were analysed for UL138 and IE1 gene expression by RT-qPCR. All samples were normalised to GAPDH. (B) Relative expression of UL138 and IE1 gene expression. Data presented are pooled from 3 biological replicates, with bars representing the averages of triplicate measurements, and standard deviations shown. Statistical analysis was by one-way analysis of variance (ANOVA) (UL138: $F = 43.30$, degrees of freedom, d.f. = 11, $P < 0.0001$; IE: ($F = 1073.27$, d.f. = 11, $P < 0.0001$), using a post-hoc Tukey's multiple comparison test. Results generated from LUNA knockout viruses are significantly different from WT phenotype controls.

4.2.1. Primary CD14$^+$ monocytes line support latent HCMV infections in vitro

While primary CD34$^+$ cells serve as a robust model for supporting experimental HCMV latency, they are expensive to obtain on a regular basis. For this reason, more easily obtainable types of primary myeloid cells, capable of supporting latency, were used to further evaluate the role of LUNA.

To validate primary CD14$^+$ monocytes as an alternative cell model for this investigation, monocytes infected with wild-type Merlin virus were co-cultured with fibroblasts to monitor production of infectious virus. Prior to infection, monocytes were either left untreated (undifferentiated) or pre-treated with prednisolone (causing myeloid differentiation)[316]. After 7 days post-infection, no viral UL32-GFP-expressing foci were detected in co-cultures containing undifferentiated monocytes, indicating successful latency establishment; however, in those that had been treated with prednisolone, evidence of infectious viral spread was observed, consistent with the acquisition of a permissive phenotype that accompanies cellular differentiation (Figure 4.3).

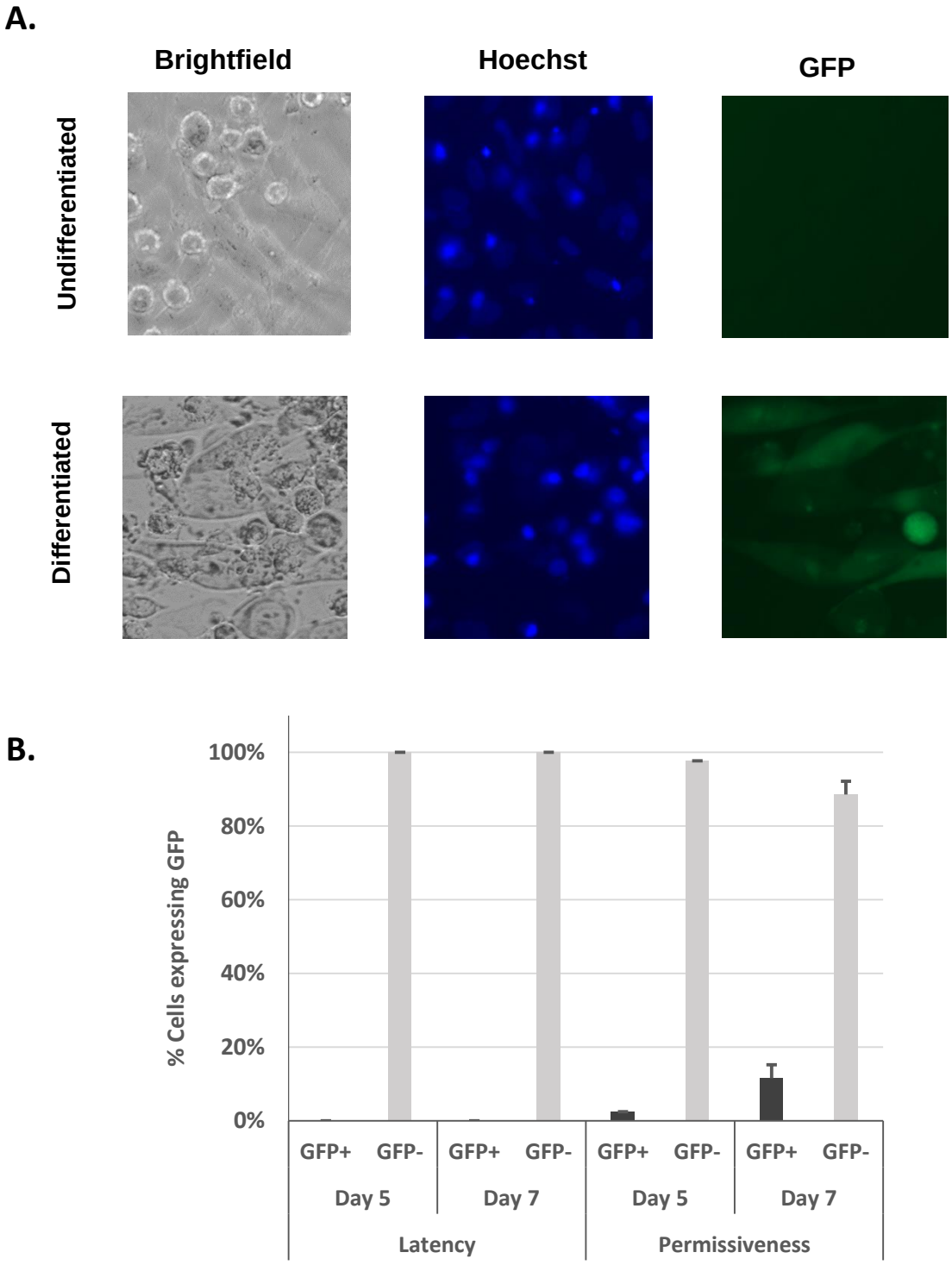


Figure 4.3. Undifferentiated CD14^{+} monocytes support latent HCMV infection

CD14^{+} monocytes isolated from healthy individuals were plated at 5×10^{5} cells per well and cultured for 4 days in the presence of prednisolone (differentiated) or under mock conditions (undifferentiated) prior to infection with wild type UL32-GFP-tagged Merlin virus at MOI = 5. (A) Cells were visualised for GFP expression at 5 and 7 days post-infection. (B) The number of GFP positive or negative cells were enumerated using ImageJ software. Bars represent averages of 5 fields of view of 100 cells, each showing standard deviation.

4.2.2. LUNA is required for efficient viral gene expression during latent infection of primary CD14$^+$ monocytes

Earlier, I showed that LUNA appears to be required for efficient viral gene expression during HCMV latency in CD34$^+$ cells. To determine if this CD14$^+$ model of experimental latency also demonstrates a similar requirement for LUNA, total RNA was extracted from CD14$^+$ monocytes infected with LUNA deficient virus, then assayed for UL138 and IE1 expression as before. Consistent with the previous trend, an approximate five-fold decrease in the levels of both UL138 and IE gene expression were observed during latent infection of CD14$^+$ monocytes with LUNA deletion virus compared to wild type revertant (Figure 4.4).

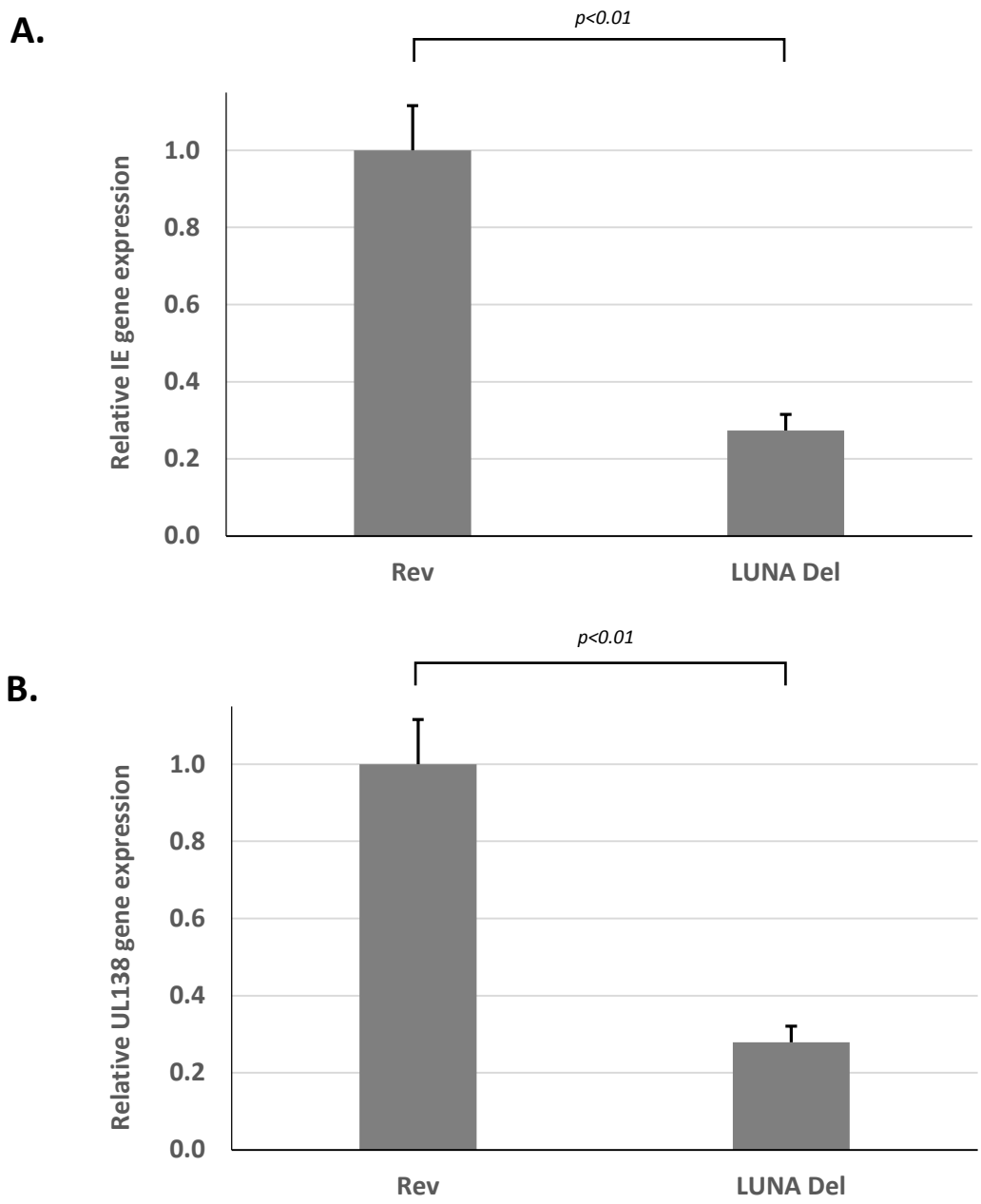


Figure 4.4. LUNA is required for efficient viral gene expression in latently infected CD14^{+} monocytes

(A) RNA isolated from CD14^{+} monocytes plated at 5×10^{5} cells per well latently infected with revertant (Rev) and LUNA deletion (LUNA Del) viruses at MOI = 5. After 3 days post infection, the cells were harvested and analysed for IE1 gene expression by RT-qPCR. All samples were normalised to GAPDH. (B) As above, except for UL138 expression. Data presented are pooled from 3 biological replicates, with bars representing the averages of triplicate measurements, and standard deviations shown. Student's *t* test was used to determine the significance of differences between gene expression of Rev and LUNA KO infections.

4.2.1. LUNA-associated changes in viral gene expression correlate with histone protein modifications during HCMV latency

To provide a possible molecular explanation for the differences in levels of viral gene expression observed during latent infection with HCMV, ChIP analyses were performed in latently infected monocytes to evaluate the epigenetic landscape surrounding latency-associated viral gene promoters in particular. The viral latency-associated genes *UL144* and *LUNA* itself were chosen as candidates for interrogation, owing to the fact that their promoter sequences had already been functionally characterised[282]. Both UL144 and LUNA are important for the efficient establishment of latency in an *in vitro* system. Moreover, although UL144 is known to be expressed in a strain-dependent manner, Merlin virus is known to express UL144 during latency[282].

Briefly, with respect to epigenetic profiling, the 'nucleosome-code' hypothesis posits that the post-translational modification of histones is associated with the regulation of gene expression, either by relaxing or condensing the local chromatin structure to activate or repress transcription, respectively[166]. For instance, promoters of recently transcribed or active genes are denoted by the presence of histone H3 trimethylated at lysine 4 (H3K4me3), which is otherwise absent from silenced genes. By contrast, repressed genes and heterochromatin are linked to the presence of H3K9me3. Measuring H3K4me3 enrichment at the host gene *TLR4* and H3K9me3 at *ZNF180* serve as positive controls for activatory and repressive marks, respectively, in myeloid cells.

In keeping with the transcriptional data shown above, observable changes in histone modification patterns around latency-associated promoters appeared to reflect their own promoter activity. Specifically, LUNA expression during a latent infection coincided with the acquisition of markers of transcriptional activation over the promoters of latency-associated viral genes (Figure 4.5A). Additionally, a converse trend showing a loss of markers of transcriptional repression from the same promoters was observed in the presence of LUNA, however, no statistical significance was ascertained for the UL144 promoter and UL138 promoter (Figure 4.5B).

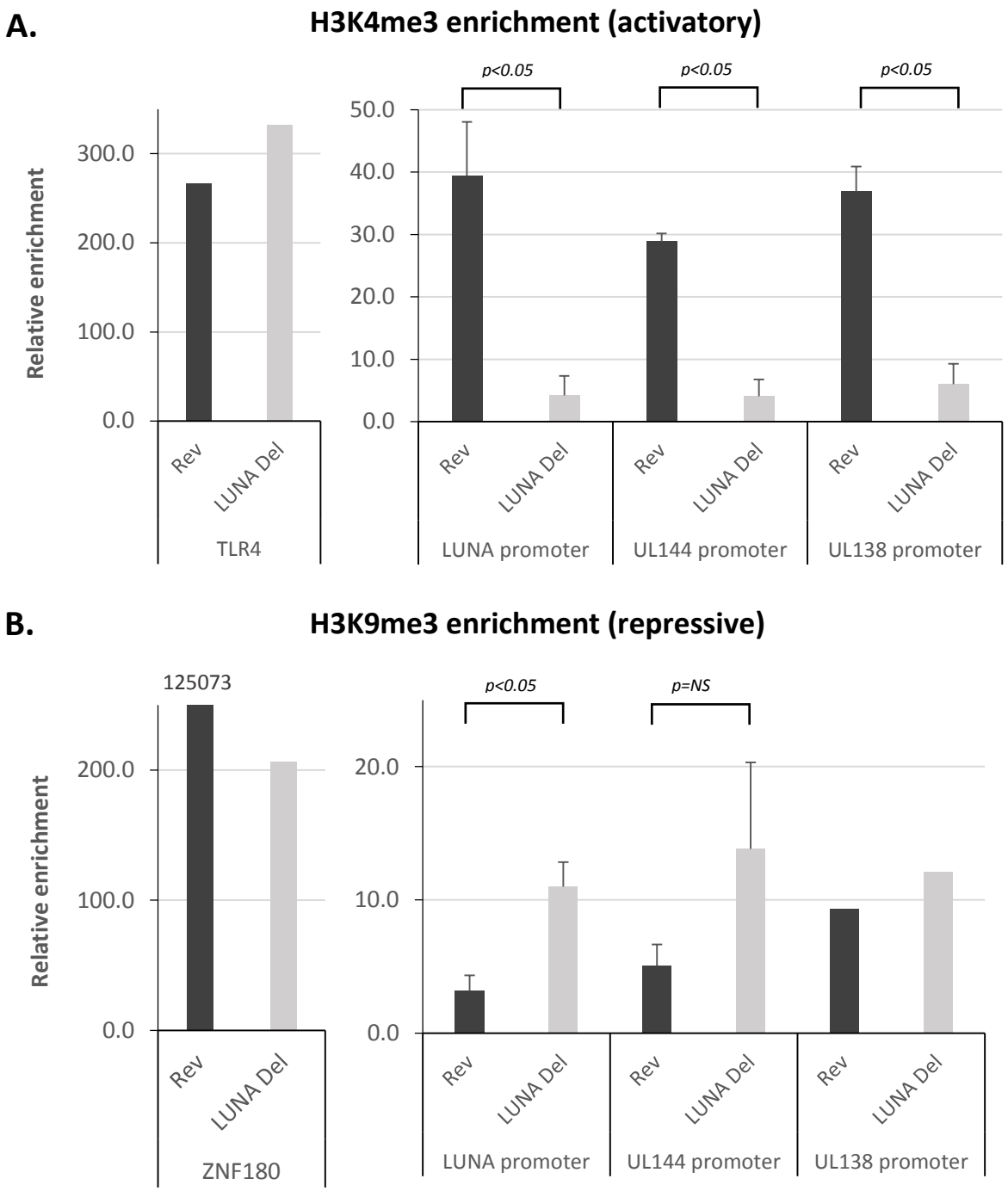


Figure 4.5. LUNA promotes changes in histone modification patterns during latency

Chromatin immunoprecipitation assays with anti-histone H3 trimethylated at lysine 4 (H3K4Me3), anti-H3K9Me3, or isotype control (IgG) antibodies were performed on 1×10^7 $CD14^+$ cells latently infected with revertant (Rev) or LUNA deletion (LUNA Del) virus at MOI = 4. Samples were amplified using primers targeting known regions in the LUNA promoter and UL144 promoter and expressed as a logarithmic function over the Input. H3K4me3 enrichment is represented in **A.** TLR4 was used as a positive control candidate for H3K4me3 enrichment. H3K9me3 enrichment is represented in **B**. ZNF180 was used a positive control candidate for HEK9me3 enrichment. Bars represent averages of triplicate analyses from two independent experiments, with standard deviation shown. Student's *t* test was used to determine the significance of the differences in histone enrichment between infections with Rev and LUNA Del viruses. Points with no error bars represent averages of triplicate measurements from a single experiment.

4.2.2. LUNA is required for efficient viral reactivation during latent infection of primary CD34$^+$ progenitors

So far, this Chapter has shown that the expression of LUNA is required for efficient latency-associated viral gene transcription during latent infection. Because viral gene expression during latency is essential for maintaining the latent phenotype, it is plausible that LUNA may contribute towards the process of latency by promoting gene expression in the latently infected cell. To evaluate this further, our laboratory performed an experiment in collaboration with Matthew Reeves (UCL) to examine whether LUNA was required for viral reactivation from latency following myeloid cell differentiation.

Primary CD34$^+$ cells were latently infected with LUNA-deficient Merlin virus and subsequently differentiated to dendritic cells 7 days post-infection. Cells were then stimulated with LPS to induce reactivation, and in turn analysed for IE gene expression by RT-qPCR and assayed for infectious virus production by co-culturing with human fibroblasts. It was observed that the induction of IE gene expression was impaired in cells infected with LUNA deletion mutants and that this was consistent with a reduction of reactivation of infectious virions (Figure 4.6A and 4.6B). Together, this indicates that LUNA is also important for enabling HCMV to undergo reactivation after it has established latency.

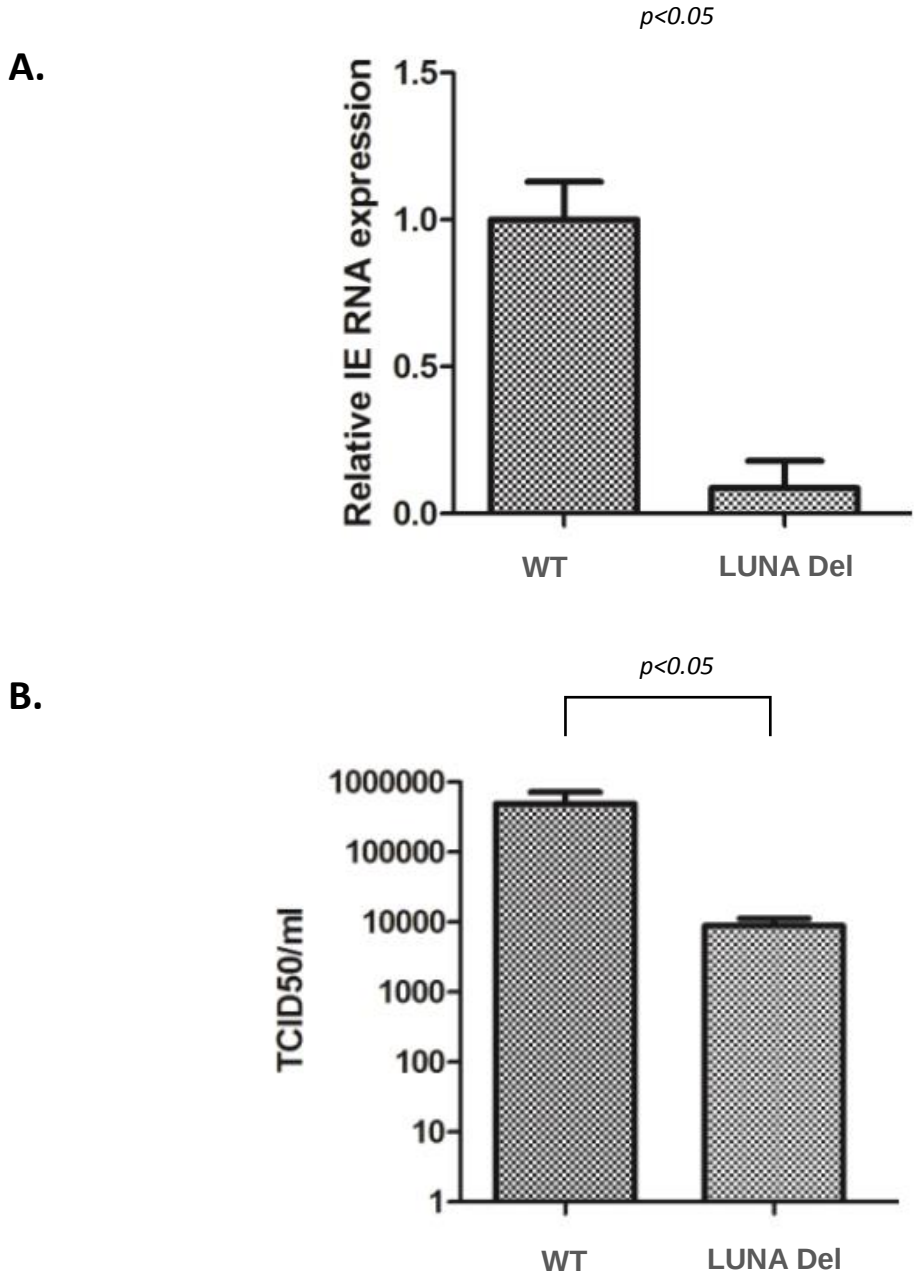


Figure 4.6. LUNA is required for efficient reactivation of HCMV following differentiation of latently infected early myeloid cells (Figure courtesy of Emma Poole)

Primary $CD34^+$ cells, plated at 1×10^5 cells per well, were latently infected with WT and LUNA Del viruses at MOI = 5. At 3 days post-infection, cells were differentiated to immature dendritic cells by the addition of differentiation cytokine media. At 10 days post-infection, cells were then incubated with LPS for 24h to promote dendritic cell maturation and hence reactivation. Cells were then analysed for IE gene expression by RT-qPCR (A) or co-cultured with HFFs and assayed for infectious virion production (B). Bars represent averages of triplicate analyses from two independent experiments, with standard deviation shown. Student's *t* test was used to determine the significance of the differences in histone enrichment between infections with WT and LUNA Del viruses.

4.3. DISCUSSION

Together, these findings demonstrate that the HCMV LUNA protein plays a role in regulating viral gene expression during latency. This is evidenced by the reduced detection of viral gene transcripts in models of experimental latent infection using viruses deficient in LUNA (Figures 4.2 and 4.4). The observed differences in viral gene expression also coincided with corresponding changes in the chromatin structure of latency-associated genes, suggesting a possible molecular basis for their regulation (Figure 4.5). Moreover, on a functional level, it also appears that LUNA is required for enabling viral reactivation from latency (Figure 4.6).

Since LUNA appears to contribute towards the control of viral gene expression during latency, this fits with previously published work demonstrating its importance in maintaining latent genome carriage in myeloid cells. Notably, UL138 is a latency-associated gene product whose expression is required for maintaining virally-infected cells in a latent state[260]. When LUNA is absent during latency, the expression of UL138 drops significantly as a result, which supports LUNA having a putative role in latency establishment. Additionally, levels of MIE gene expression were also positively upregulated by LUNA during latency. This is surprising as in latently infected cells, the MIEP (which controls expression of IE1 and IE2) assembles into repressed chromatin through a process dependent on the UL138 gene product[285]. Given that LUNA promotes the disruption of ND10, which are known intrinsic repressors of HCMV IE gene expression, one hypothesis is that LUNA may augment the activity of the MIEP by affecting the overall balance of transcriptional regulators which target it. Thus, it is also conceivable that by allowing the MIEP to be maintained in a more poised state for transcriptional activation, LUNA may also be required for latent genomes to reactivate efficiently, once a sufficient stimulus for reactivation is given. Work performed in collaboration with Matthew Reeves has confirmed this, whereby latently infected $CD34^+$ cells fail to efficiently express IE genes or undergo lytic replication following terminal differentiation to mature DCs when LUNA is not present.

Data obtained from ChIP analysis also show that LUNA can affect the chromatin landscape over viral genes during latency. Clearly, this suggests a potential epigenetic mechanism by which LUNA can affect levels of viral gene transcription. It is therefore plausible that the expression levels of gene transcripts for whose promoters were not assayed by ChIP would also be similarly affected in latently infected cells when LUNA is absent. Notably, the finding that the promoters of latency-associated genes showed a clear switch between the enrichment

of repressive (H3K9me3) and active (H3K4me3) marks points to a dynamic regulation and it would interesting to further evaluate the which proteins or chromatin modifiers bind to those promoters and the impact LUNA has in determining their presence or absence.

An oversight worth noting in this investigation is the absence of available data demonstrating equal infectivity of each virus, particularly in support of results shown in Figures 4.2 and 4.4. Understandably, this impacts on the interpretation of the role of LUNA on latency-associated viral gene expression, whereby reduced levels of virus gene expression seen during latent infection with LUNA deletion virus compared to WT phenotype viruses may reflect low levels of latent viral genome carriage and associated gene expression, rather than lack of an upregulatory effect from LUNA. Although appropriate methodological steps were taken to ensure equal infectivity (e.g. uniform numbers of cells plated and calculating the respective PFUs for each experiment), data produced from an ongoing study that utilises the same virus materials described above has shown that equal MOI infection of myeloid cells with LUNA deletion and LUNAg233c mutant (described later) resulted in equivalent establishment of latency[317].

Overall, however, it remains unclear whether the totality of the effects described are driven by the ability of LUNA to disrupt ND10 in the latently infected cell. Despite this plausibility, further work is required to demonstrate whether the disruption of ND10 (by LUNA) is responsible for affecting changes in the local chromatin structure at the viral DNA that would in turn correspond with the regulation of viral gene expression downstream.

5. Evaluating the direct impact of LUNA expression on the activity of latency-associated viral gene promoters

5.1. INTRODUCTION

In the previous Chapter, I established that virus gene expression during latent HCMV infection is regulated by the viral LUNA protein. Thus, when undifferentiated myeloid cells become infected with LUNA-deficient viruses, fewer latency-associated gene transcripts (as well as IE transcripts) are detected.

One of the ways in which LUNA may regulate the expression of HCMV genes is by stabilising nascent RNA so that levels of viral messenger transcript are maintained throughout the duration of infection. However, given that the expression of LUNA in the latently infected cell correlates with the establishment of active chromatin over the promoters of latency-associated viral genes, it seems more likely that LUNA functions instead by activating viral gene transcription. Therefore, under latent conditions, one may hypothesise that the promoters of latency-associated genes would exhibit a difference in activity depending on whether LUNA was present or absent. As such, the aim of this investigation was to test the above hypothesis in order to further evaluate the role of LUNA in controlling viral gene expression during latency.

5.2. RESULTS

5.2.1. The myelomonocytic Kasumi-3 cell line supports HCMV latency in vitro

Before assessing the ability of LUNA to regulate viral gene expression at the transcriptional level, it was first necessary to develop a tractable model system with which to study such effects of LUNA *in vitro*. Much of the data I have presented so far, in respect of functionally characterising LUNA, has been obtained using primary myeloid cells that are fully capable of supporting HCMV latency. However, these cell types are difficult to manipulate by molecular techniques (e.g. transfection), rendering them unsuitable for use in this study. Therefore, the $CD34^+$ haematopoietic progenitor cell line Kasumi-3 was selected as an alternative model, as these cells are easily manipulatable and faithfully reproduce key aspects of latent HCMV infection[312,318,319]. These cells allow for an HCMV infectivity rate of up to 11% at high MOI, include the ability to maintain viral genome carriage in the relative absence of lytic IE gene expression, and also permit the expression of latency-associated genes, such as LUNA, following infection with clinical isolates of HCMV[312]. In addition, unlike THP-1 cells, which can also support the above aspects of latency, Kasumi-3 cells have the noted ability to support full reactivation from latency, resulting in new virion production[312].

To confirm that Kasumi-3 cells supported latent infection and productive replication in this investigation, cells were infected using the low-passage strain TB40-BAC4, which expressed the lytic MIE gene IE2 fused to red fluorescent protein (RFP). After 3 days post-infection, no detectable IE2 protein was present, however these cells were still able to reactivate upon differentiation (Figure 5.1). Additionally, cells that were differentiated prior to infection exhibited a permissive phenotype for lytic infection (Figure 5.1). These results are consistent with findings obtained from previous analyses of IE gene expression in these cells[312].

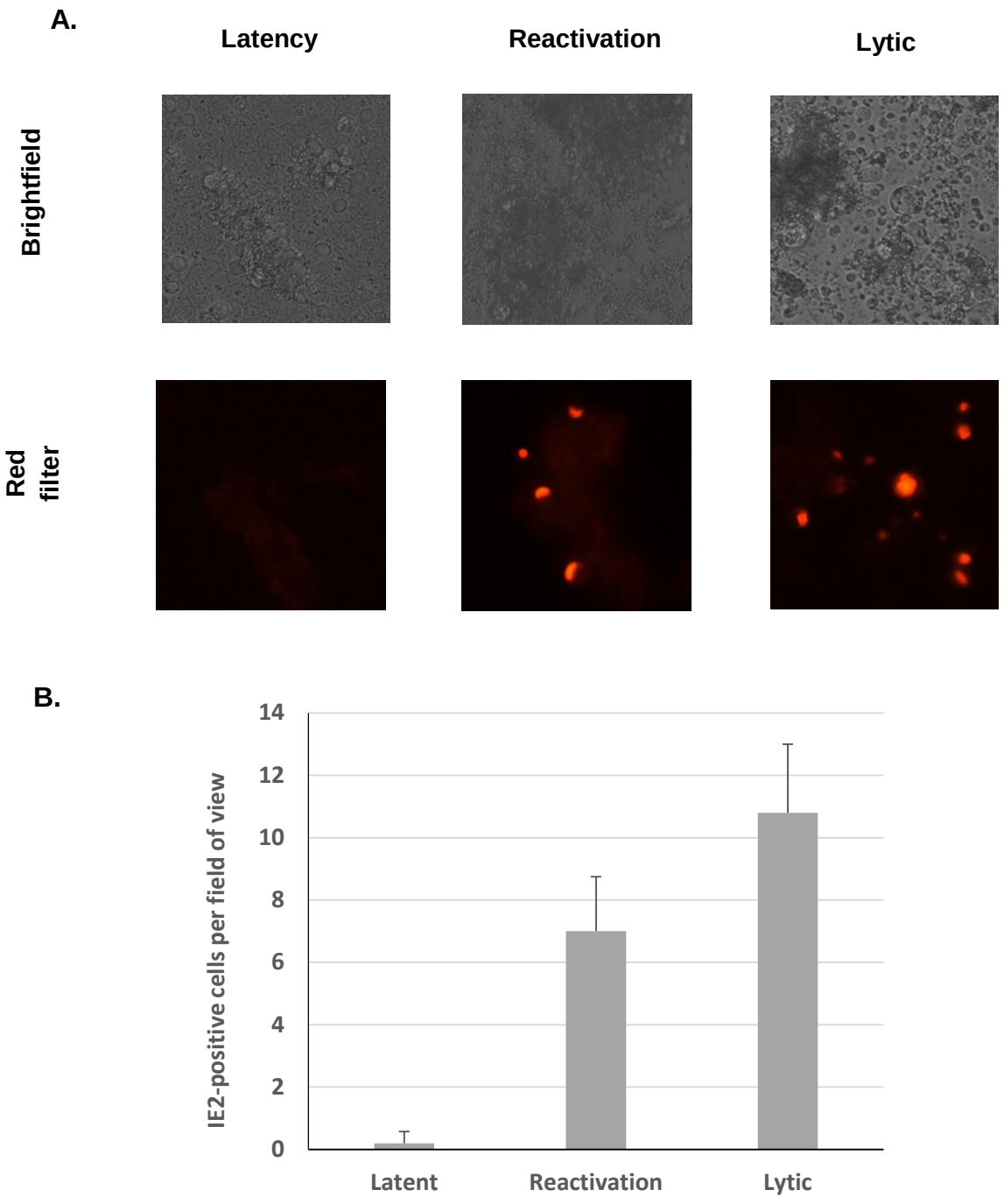


Figure 5.1. Initiation of lytic gene expression is differentiation-dependent in Kasumi-3 cells

Undifferentiated Kasumi-3 cells were infected at MOI = 3 with recombinant TB40-BAC4 strain ectopically expressing RFP fused to IE2 and incubated for 3 days prior to being analysed (latency). Following the 3-day period of latency establishment, the same samples were treated with phorbol 12-myristate 13-acetate for 48h to promote differentiation before analysis (reactivation). Additionally, separate cultures of Kasumi-3 cells were pre-treated with phorbol 12-myristate 13-acetate for 48h prior to being infected (lytic). Cells were analysed directly by immunofluorescence, presented graphically (A) and enumerated in (B). Data shown represent averages of triplicate samples of six fields of view. Error bars denote standard deviation.

5.2.2. Transient expression of LUNA correlates with an increase in the activity of latency-associated viral gene promoters

To examine the impact of LUNA on viral transcriptional activation, transient co-transfection assays were performed to express the protein in isolation and subsequently observe its effects on the levels of activity of latency-associated viral gene promoters derived from *UL144* and *LUNA*. Having shown that the Kasumi-3 cell line supports latent HCMV infection *in vitro*, these cells were subsequently used as a model system for homologous expression so as to provide a cellular context that was as closely related to latency establishment as possible.

Consistent with the changes in virus gene expression observed earlier at the mRNA level, the expression of wild-type LUNA routinely resulted in increased levels of transcriptional activation from the UL144 promoter and LUNA promoter (compared to empty transfected controls) (Figure 5.2).

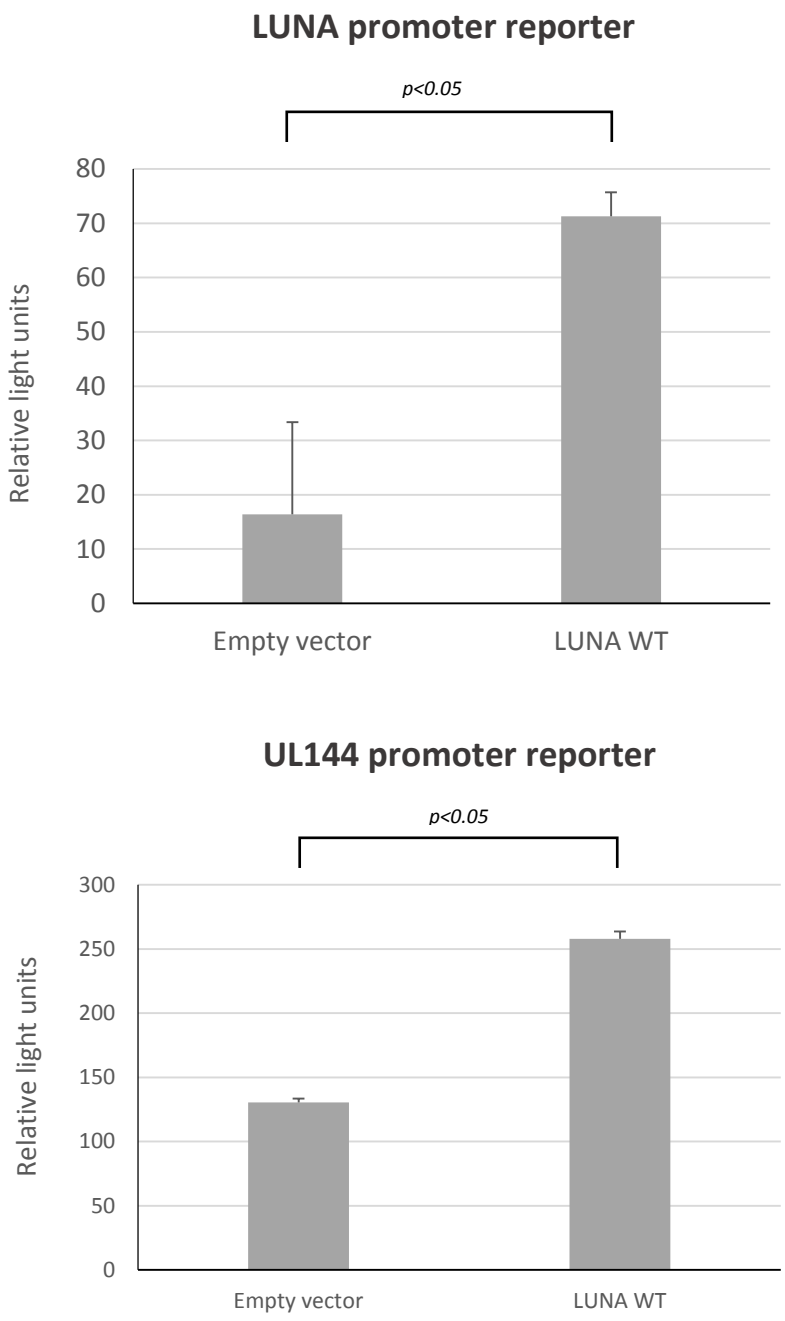


Figure 5.2. Transient overexpression of LUNA activates the promoters of latency-associated viral genes

The $CD34^+$ myeloblastic cell line Kasumi-3 was transiently co-transfected with luciferase reporter plasmids (pGL3) under the control of latency-associated viral gene promoters and effector expression vectors (pCMV-Tag2b) containing FLAG-tagged wild type LUNA (WT). Cells were harvested 48 hours post transfection, then assayed for luciferase expression. Results are given as background-subtracted relative light units. Data presented are pooled from 3 biological replicates, with bars representing the averages of duplicate measurements, and standard deviations shown. Student's *t* test was used to determine the significance of differences in promoter activity between Empty and LUNA WT transfections.

5.2.1. Knockdown of major ND10 component PML in combination with direct expression of LUNA exerts a positive effect on the activation of UL144 promoter, but not LUNA promoter

The ability of LUNA to augment the activation of latency-associated viral gene promoters, as shown above, may be linked to the disruption of ND10 structures and the delocalisation of host restriction factors from viral genomes. This is plausible given that it has already been shown that ND10 are disrupted following the isolated expression of LUNA in Kasumi-3 cells. To determine whether ND10 are directly involved in affecting the ability of LUNA to augment the activity of latency-associated viral gene promoters, the levels of LUNA-mediated promoter activation were assayed in the absence of PML using RNA interference knockdown. THP-1 cells recombineered to express shRNA to PML were used as the expression system since they have also been shown to act as a tractable model of latent infection in previous studies.

Despite no statistical significance when the data are interrogated, a trend is nonetheless observed whereby in the presence of cellular ND10 (siC), the LUNA promoter is activated following co-transfection of the LUNA gene, but such a trend is not observed in cells that have already had ND10 knocked down by siPML (Figure 5.3A). This supports the notion that LUNA functions to activate gene expression, insofar as the LUNA promoter is concerned, by disrupting ND10. Notably, while it is unclear why a greater magnitude of responsiveness to LUNA expression was seen in siC cells compared to siPML, one may posit that the different properties between the two cell types may have impacted on the final outcome of the assay. The UL144 promoter is different in that, again although not statistically significant, a trend is observed showing that this promoter is activated by LUNA co-transfection in cells with PML bodies; however in cells devoid of ND10 that activation is more substantial and is statistically significant (Figure 5.3B). Thus, at least for the UL144 promoter, LUNA appears to act in a PML independent fashion. Regardless, it is clear that further repeats of this experiment are necessary to determine if the effects of LUNA are drawn primarily from their ability to disrupt PML since one would expect to see a significant difference between LUNA transfections compared to empty transfection controls in cells where ND10 are intact, but not where ND10 are absent.

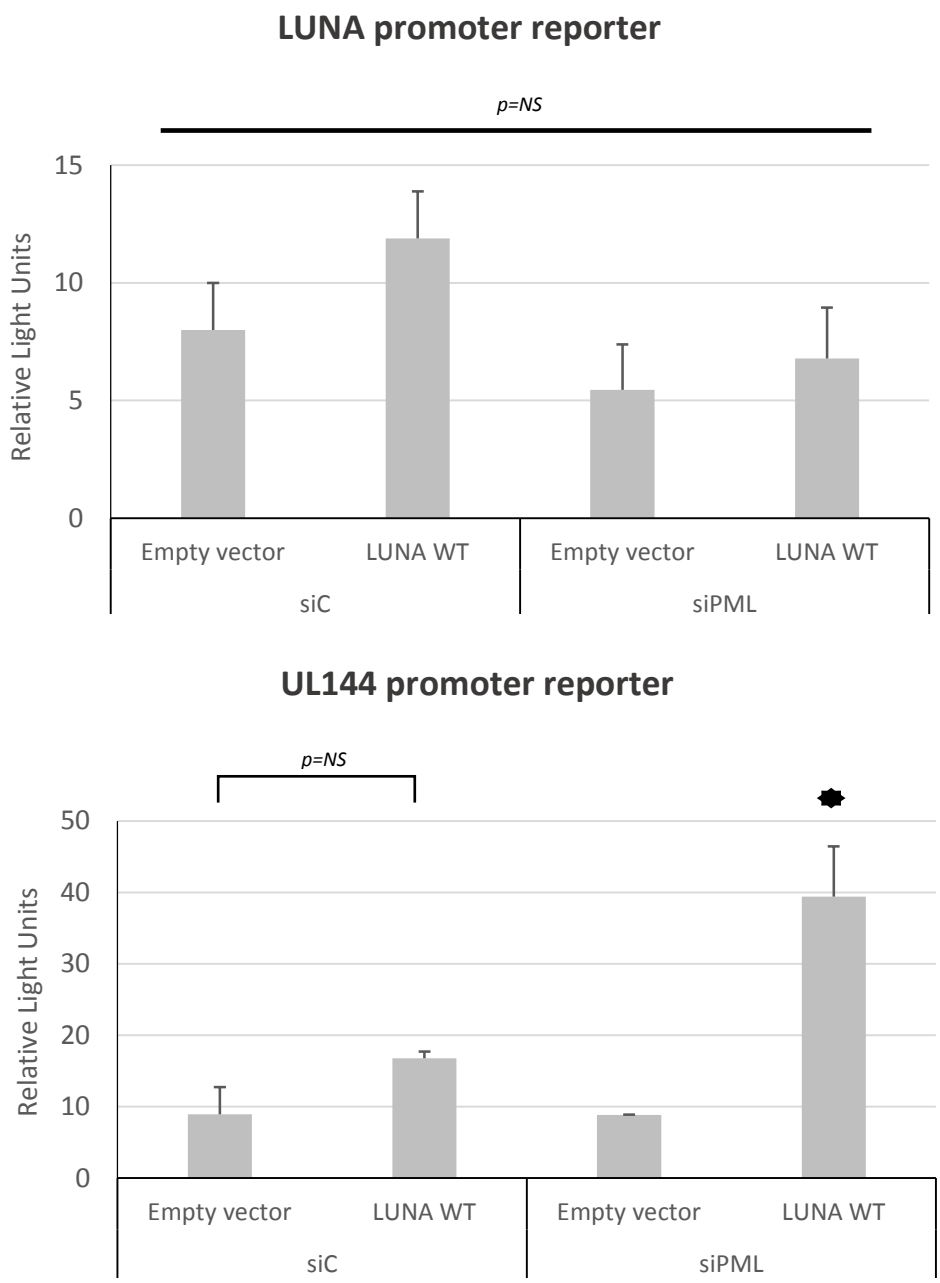


Figure 5.3. In the context of PML protein depletion, only the UL144 promoter, but not the LUNA promoter, is responsive to LUNA protein expression

THP-1 cells stably expressing either functionally inactive shRNA (siC) or shRNA to PML (siPML) were transient co-transfected with with luciferase reporter plasmids (pGL3) under the control of latency-associated viral gene promoters and effector expression vectors (pCMV-Tag2b) containing FLAG-tagged wild type LUNA (WT). Cells were harvested 48 hours post transfection, then assayed for luciferase expression. Results are given as background-subtracted relative light units. Data presented are pooled from three biological replicates, with bars representing the averages of duplicate measurements, and standard deviations shown. Statistical analysis was by one-way ANOVA (LUNA reporter: $F = 1.44$, d.f. = 11, $P > 0.01$; UL144 reporter: $F = 15.70$, d.f. = 11, $P < 0.01$), using a post-hoc Tukey's multiple comparison test. For samples containing LUNA reporter, no statistical significance was observed between any of the groups. For samples containing UL144 reporter, only results generated from transfection of LUNA expression vectors into siPML cells were statistically significant compared to all other groups (*).

5.2.2. LUNA and the host transcription factor GATA-2 cooperate to exert an additive effect on the activation of latency-associated viral gene promoters

Previous reports have shown that the LUNA and UL144 promoters possess binding sites for the host transcription factor GATA-2, which has been implicated in promoting the expression of these genes during latency[215]. To determine whether the viral LUNA protein itself may aid GATA-2-mediated transactivation of latency-associated viral gene promoters, both LUNA and GATA-2 were introduced into the same reporter expression system developed in Kasumi-3 cells to measure their possible combined effect on latency-associated promoter activation.

As before, the activities of the LUNA promoter and UL144 promoter were upregulated in response to the sole expression of wild-type LUNA (Figure 5.4). This effect was also similarly observed in the case of GATA-2, which is consistent with previous published data demonstrating that both promotors – which had been derived from the low-passage strain Merlin – are responsive to GATA-2 (Figure 5.4). Notably, however, the co-expression of both LUNA and GATA-2 resulted in a cooperative, additive effect on the activation of both promoters (Figure 5.4).

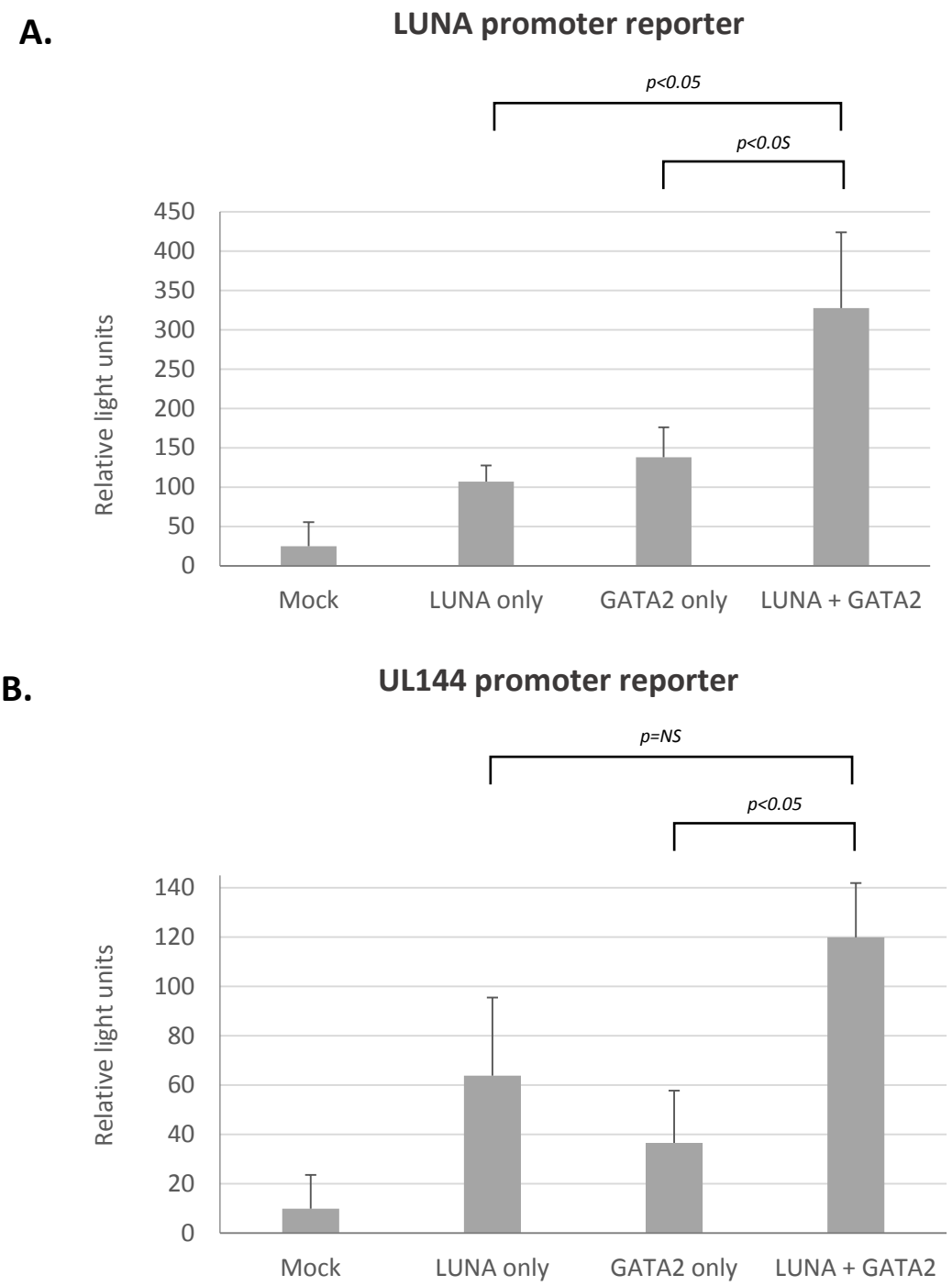


Figure 5.4. Co-transfection of LUNA and GATA-2 exerts a combined, additive effect on the activity of latency-associated viral gene promoters

Kaasumi-3 cells were co-transfected for 48h with luciferase reporter plasmids (pGL3) under the control of the LUNA promoter (A) or UL144 promoter (B), along with effector vectors pCMV-Tag1A containing empty (Tag1A) or Triple-Flag-tagged wild type LUNA (LUNA); and pEF3.1 containing empty (pEF) or wild type GATA-2 (GATA-2) cDNA. Results are presented as background-subtracted relative light units (RLU). Data were pooled from 3 biological replicates, with bars representing the averages of duplicate measurements, and standard deviations shown. Student's *t* test was used to determine the significance of differences in promoter activity between LUNA + GATA-2 against LUNA only and GATA-2 only transfections.

5.2.3. LUNA exhibits a physical interaction with GATA-2 in vitro

The viral latent gene product LUNA and host transcription factor GATA-2 appear to work cooperatively to facilitate the transcriptional activation of latency-associated viral genes, insofar as *LUNA* and *UL144* are concerned. To this end, a physical protein-protein interaction might exist between LUNA and GATA-2, which might be important to enable their combined functionality.

Following pull-down of FLAG-tagged LUNA with anti-FLAG antibody, GATA-2 was detected using anti-GATA-2 antibody by Western blotting of lysates from HEK293T cells simultaneously co-transfected with FLAG-LUNA and GATA-2 expression vectors (Figure 5.5). This indicates that a physical interaction exists between the two proteins. Notably, the band for GATA-2 was seen at approximately ~110kDa, indicating that the protein may have undergone some form of post-translational modification following transfection.

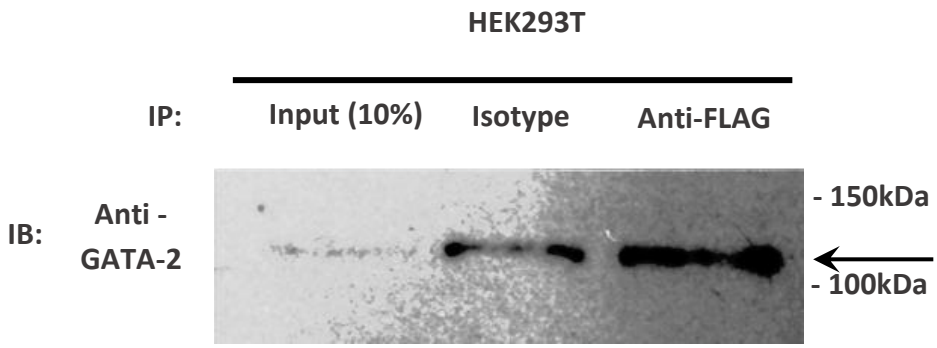


Figure 5.5. Viral LUNA protein interacts with host transcription factor GATA-2 *in vitro*

Triple-FLAG-tagged LUNA (LUNA) was immunoprecipitated with anti-FLAG rabbit IgG antibodies from HEK293T cells co-transfected with expression vectors containing GATA-2 and LUNA. Cell lysates before immunoprecipitation (Input) and immunoprecipitates (IP) were analysed by SDS-PAGE and immunoblotting (IB) with indicated antibodies. Arrow indicates the position of GATA-2. To further substantiate this finding, a reverse-pulldown was carried out, but was unsuccessful.

5.3. DISCUSSION

The results of this investigation demonstrate that LUNA is able to regulate the expression of latency-associated genes at the transcriptional level, specifically, by augmenting the activity of viral gene promoters which are otherwise known to be active during latency (Figures 5.2 and 5.3). Thus, consistent with the findings observed earlier in Chapter 4, these data support a potential mechanistic pathway by which LUNA may exert control over latency-associated viral gene expression.

The depletion of PML appears to impact on the ability of LUNA to activate its own promoter, consistent with the view that LUNA functions by disrupting ND10 bodies during latent infection; however, it does appear that LUNA may promote gene expression by other mechanisms besides ND10 disruption, as evidenced by the responsiveness of the UL144 promoter to LUNA expression when PML was already absent. Nevertheless, with the exception of results pertaining to the UL144 promoter in the absence of PML, the data obtained are non-significant, requiring additional repeats before any firm conclusions may be drawn outright.

Notably, the observation that LUNA expression correlates with the activity of its own promoter, indicates that LUNA is associated with the positive autoregulation of itself. If LUNA is responsible for stabilising its own expression during latency, as well as presumably that of other latency-associated gene transcripts (e.g. UL138), this raises the question as to how LUNA may come to be expressed initially upon latent infection. One possible explanation is that other factors may be involved in activating LUNA gene expression, which may then result in subsequent formation of a positive-feedback loop affecting other promoters. Consistent with this, binding of the host transcription factor GATA-2 to the LUNA promoter has previously been reported, and here, has been shown to increase LUNA promoter activity in transfection assays[215]. Interestingly, the combined presence of LUNA and GATA-2 also yields an additive effect on transcriptional activation (Figure 5.4). In support of their ability to cooperate together, both LUNA and GATA-2 appear to demonstrate a physical interaction with each other (Figure 5.5).

6. Evaluating the impact of a LUNA intrinsic deSUMOylase activity upon the regulation of viral gene expression during latency

6.1. INTRODUCTION

Recent protein sequence analysis of LUNA, performed in collaboration with Dr Matthew Reeves (UCL), has revealed that the protein possesses short amino acid homology to cellular enzymes known to be involved in the ubiquitin-like modification of cellular proteins (Poole et al. Under review). In particular, this homology resembles the active sites of known isopeptidases capable of deSUMOylating protein targets. Because SUMOylation of PML is well documented to be important for ND10 integrity, LUNA-mediated deSUMOylation of PML might explain how it is able to disrupt ND10 following its expression *in vitro*. Consequently, on this basis alone, LUNA would be predicted to be able to contribute towards the regulation of HCMV gene expression during latency. Here, evidence will be presented to demonstrate the extent to which the function of LUNA, as characterised so far, is dependent on its ability to act as a deSUMOylase.

6.2. RESULTS

6.2.1. LUNA encodes a candidate enzymatic motif that exhibits an isopeptidase activity

Bioinformatics analysis of LUNA was first performed using InterPro-scan (InterPro; EMBL-EBI) to identify any homology with known protein families. This revealed minimal sequence homology with host cellular proteins, but nonetheless reported a possible identity with ubiquitin-like modifying enzymes. Because ubiquitin-like modifiers typically possess a catalytic active site within their C-terminal domain, subsequent analysis of LUNA focussed on amino acids 70-133. Using the above sequence as a query, BLAST (NCBI, Bethesda, MD) searches of public databases showed similarity between LUNA and the C-terminal catalytic domain of the human Senp family. This family belongs to a group of so-called SUMO-specific isopeptidases that catalyse the removal of SUMO conjugates from protein substrates, employing a common hydrolytic mechanism that makes use of a conserved active cysteine residue[320]. In support of its classification as a potential isopeptidase, further sequence analysis of LUNA uncovered a candidate conserved enzymatic motif in its C-terminal domain that featured a critical cysteine residue flanked by glycine and aspartic acid (Figure 6.1). The motif itself shares homology with the yeast Ulp1 (ubiquitin-like protein 1) cysteinyl protease found in *Saccharomyces cerevisiae* and is conserved throughout the whole Senp family[321].

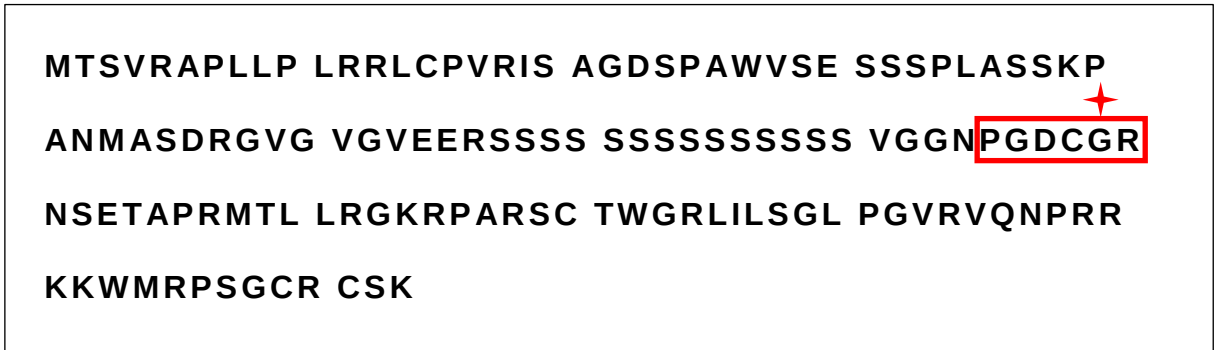


Figure 6.1. Identification of the putative catalytic site in the LUNA C-terminal domain

Bioinformatics analysis of the 133-amino-acid serine-rich LUNA protein revealed sequence similarity in its C-terminal domain with that of a group of SUMO-specific isopeptidases found in the humans. The location of the candidate conserved enzymatic motif is outlined by the red box, with the putative active cysteine residue marked by the red asterix.

In view of the predicted homology with ubiquitin-like modifying enzymes, an investigation was performed to evaluate whether LUNA was in possession of an isopeptidase activity, specifically concerning the removal of SUMO moieties from proteins (i.e. deSUMOylation). Using an enzymatic assay, levels of deSUMOylase activity exhibited by wild type LUNA were measured against that of a known cellular control, SENP2/SuPr-1 ($SENP2_{CD}$)[322]. Importantly, the analysis was extended to include the use of a LUNA mutant ($LUNA_{g233c}$), which had had the putative active cysteine changed to an inactive serine, in order to demonstrate that any catalytic activity associated with LUNA was faithful to its candidate active site (Figure 6.2A).

Purified recombinant LUNA protein showed comparable levels of activity to the control $SENP2_{CD}$ protein, suggesting that it functioned as a deSUMOylase enzyme (Figure 6.2B)[317]. Furthermore, the finding that equivalent amounts of mutant $LUNA_{g23c}$ showed no activity in the same assay, indicated that the active site encoded by LUNA was functionally responsible for conferring its deSUMOylase activity (Figure 6.2B).

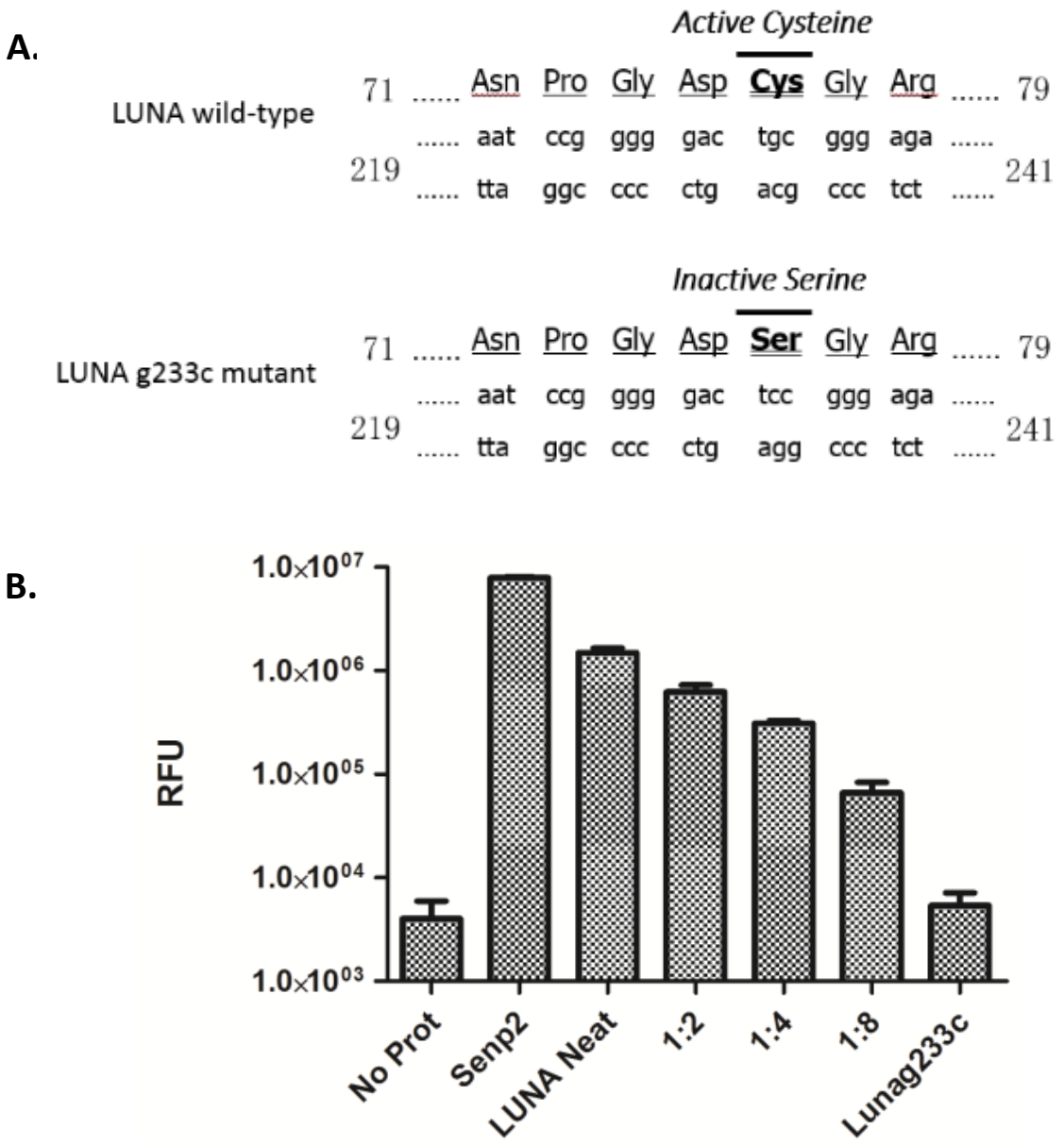


Figure 6.2. LUNA exhibits a novel deSUMOylase activity that is dependent on a putative active site featuring a catalytic cysteine residue in its C-terminal domain (Figure courtesy of Matthew Reeves)

A. The amino acid and corresponding nucleotide sequence for wild-type LUNA based on current sequence of HCMV strain Merlin. Site directed mutagenesis of nucleotide 233 was performed to convert it from a G → C, thereby changing the putative catalytic cysteine residue to an inactive serine and giving rise to the so-called 'g233c' LUNA mutant. **B.** The capacity of wild-type LUNA and the catalytically inactive g233c mutant to cleave SUMO substrate motifs was measured by fluorescence activity of a known substrate, SUMO-3, in a reporter assay. SUMO-3 was incubated with a known deSUMOylase Senp-2 (positive control), or 2-fold dilutions of wild-type LUNA or LUNA g233c mutant and the fluorescent intensity measured.

6.2.2. Disruption of ND10 by LUNA is dependent on its novel encoded deSUMOylase activity

Earlier in Section 1.1.1., transfection analyses conducted in Kasumi-3 cells showed that a greater proportion of cells were devoid of ND10 structures following the direct expression of LUNA compared to empty vector controls. Consequently, this suggested that LUNA had a role in mediating their disruption.

To determine whether the disruption of ND10 was linked to the ability of LUNA to function as deSUMOylase, use of the $LUNA_{g233c}$ catalytic mutant was also included in this analysis. Interestingly, a reverse trend was observed, whereby cells transfected with $LUNA_{g233c}$ showed hardly any evidence of ND10 disruption, with levels of intact ND10 comparable to that seen in empty vector controls, but not compared to transfection of LUNA (Figure 6.3). This finding indicates that LUNA-mediated disruption of ND10 is dependent on its ability to function as a deSUMOylase, which further supports the notion that the integrity of ND10 is susceptible to deSUMOylation. Furthermore, consistent with previous images showing WT LUNA expression in Kasumi-3 cells in Figure 3.5, $LUNA_{g233c}$ was largely localised to the nucleus. That said, I am aware that expression of the g233c mutant-FLAG tag LUNA was not assayed for differences in stability (e.g. arising from possible gross misfolding) and this will need to be evaluated in future studies.

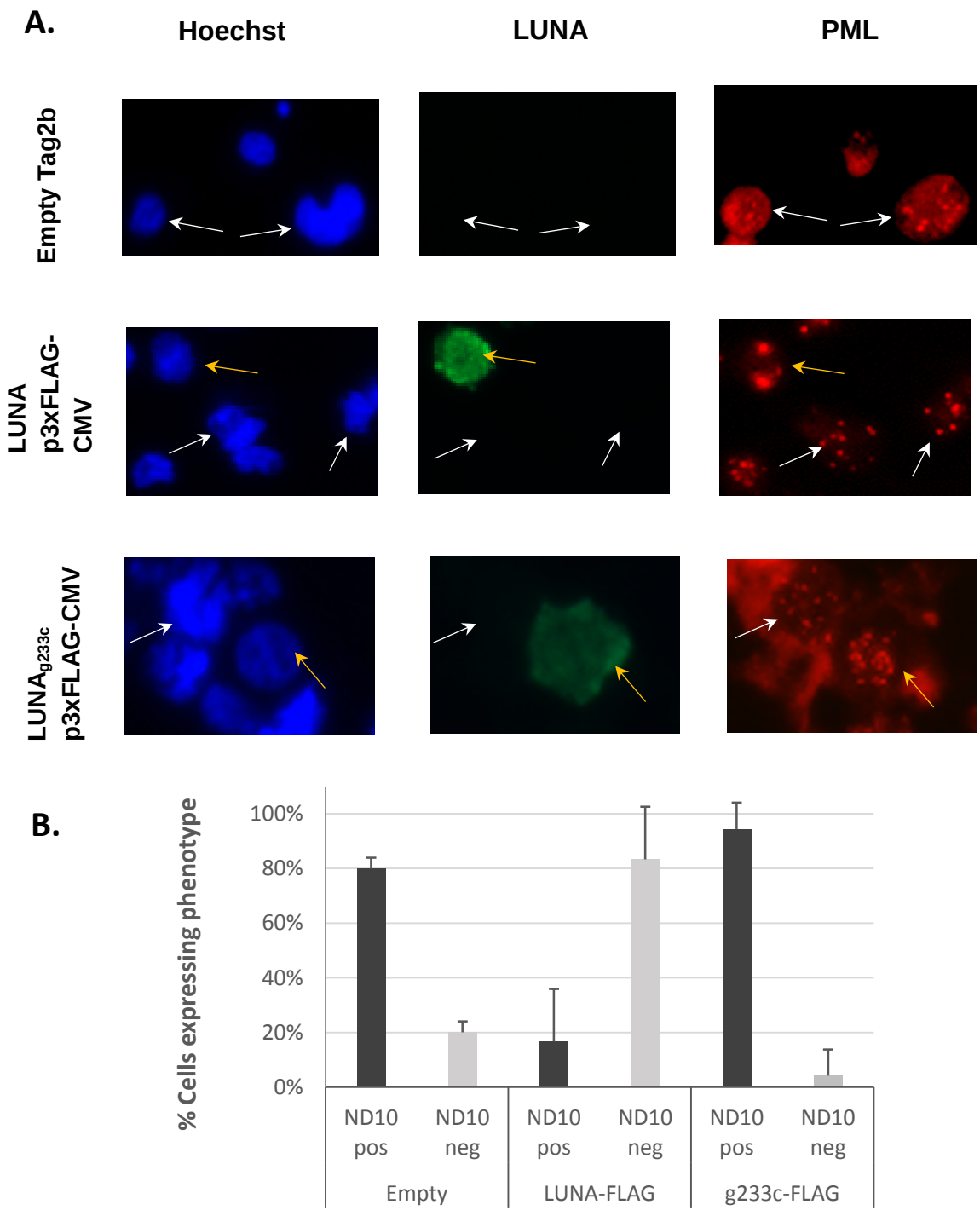


Figure 6.3. Myeloid cells transfected with LUNA$_{g233c}$ fail to show disrupted ND10

The early myeloid cell line, Kasumi-3, was transiently transfected with expression plasmids encoding wild type FLAG-LUNA (as shown earlier in Figure 4.0) or mutant FLAG-g233c fusion protein. (A) 48 hours post transfection, cells were cytospinned and stained for LUNA (green), PML (red) or nuclei (blue) (magnification; × 100). *Yellow arrows* point to a successfully infected cell exhibiting disruption of ND10 by PML staining. *White arrows* point to uninfected cells containing intact ND10. (B) The number of transfected ND10 positive or negative cells were enumerated using ImageJ software. Bars represent averages of 5 fields of view of 100 cells, each showing standard deviation.

6.2.1. Regulation of HCMV gene expression during latent infection is affected by the novel deSUMOylase activity encoded by LUNA

The results presented in Chapter 4 show that LUNA is required for efficient viral gene expression during latent HCMV infection of primary myeloid cells. Additionally, they revealed that the expression of LUNA coincided with the gain and loss of activatory and repressive histone modifications, respectively, over the promoters of latency-associated genes. Combined, the data support the view that LUNA is involved in regulating viral gene expression during latency; though, it remains unclear as to whether the deSUMOylase activity of LUNA is responsible for mediating the phenotypic changes in gene expression observed. In order to interrogate this further, the same analyses – which had been performed in latency infected $CD14^+$ monocytes – were expanded to include the use of a mutant virus (g233c), which carried the same defect in recombinant LUNA that eliminated the catalytic cysteine and was previously shown to be important for ND10 disruption in transfection analyses. This mutant did not appear to be affected in its ability to undergo lytic replication (Figure 6.4).

As with the original findings obtained using the LUNA deletion virus, the g233c virus displayed a substantial defect in its ability to induce the expression of both UL138 and IE genes during latent viral infection (Figure 6.5). Further still, latent infection with the g233c virus produced similar changes in histone modification patterns over the promoters of latency-associated genes, *UL144* and *LUNA* (Figure 6.6). Together, this suggested that LUNA mediated its regulatory effects on virus gene expression during latency through its ability to function as a deSUMOylase.

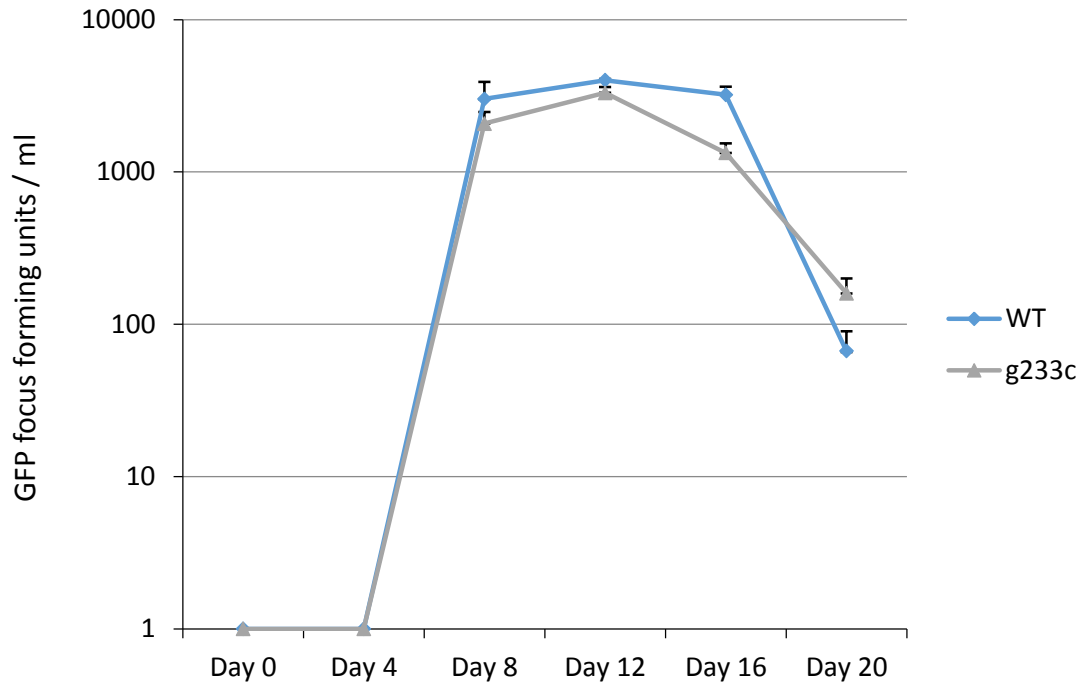


Figure 6.4. Growth of the LUNA g233c mutant virus showed no defect

HFFs were infected at an MOI of 0.01 with wild type Merlin (WT) or LUNA g233c mutant (g233c) virus and the growth measured over 10 days. Supernatants were titred for infectious virus production every 2 days as assayed by UL32-GFP foci forming units.

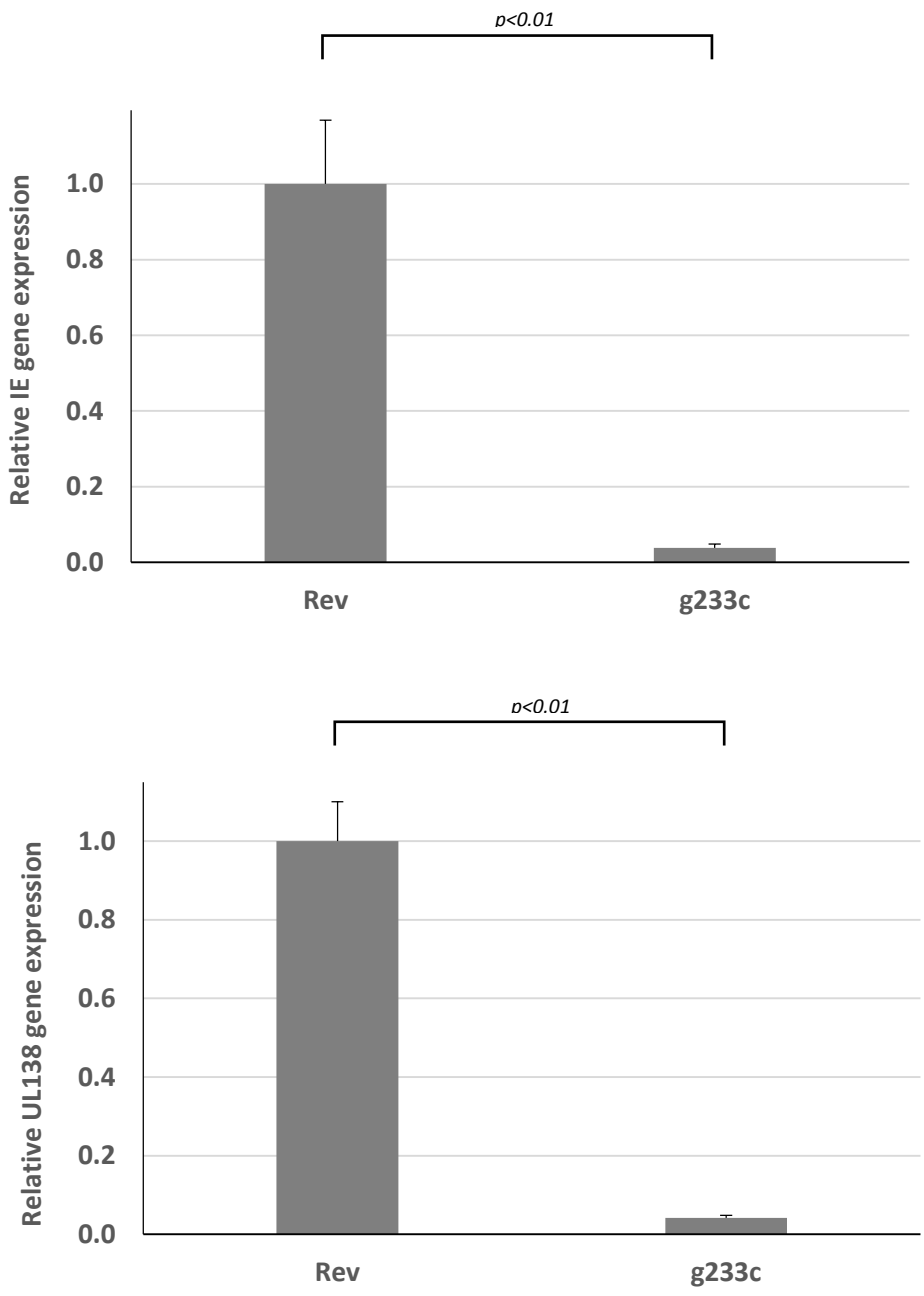


Figure 6.5. The putative deSUMOylase activity of LUNA is required for efficient latency-associated viral gene expression in latently infected CD14$^+$ monocytes

(A) RNA isolated from CD14$^+$ monocytes (5×10^5 cells per well) latently infected with revertant (Rev) and LUNA g233c mutant (g233c) viruses at MOI = 5 were analysed for IE1 gene expression by RT-qPCR 3 days post infection. All samples were normalised to GAPDH. Data presented are pooled from 3 biological replicates, with bars representing the averages of duplicate measurements, and standard deviations shown. Student's *t* test was used to determine the significance of differences in gene expression between Rev and g233c infections. (B) As above, except for UL138 expression.

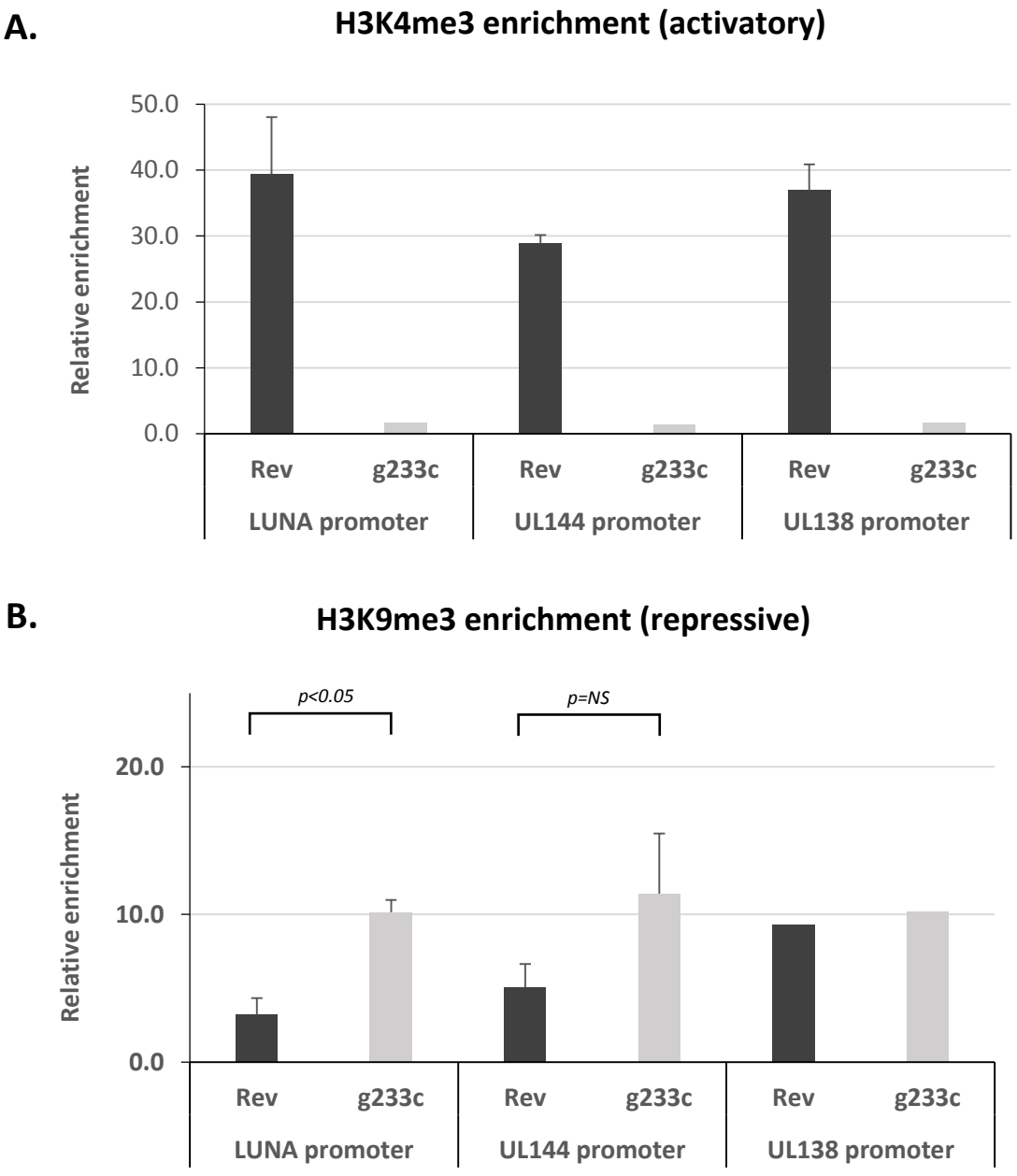


Figure 6.6. LUNA-mediated changes in histone modification patterns during latency are linked to its deSUMOylase activity

Chromatin immunoprecipitation assays with anti-histone H3 trimethylated at lysine 4 (H3K4Me3), anti-H3K9Me3, or isotype control (IgG) antibodies were performed on 1×10^7 $CD14^+$ cells latently infected with revertant (Rev) or LUNA g233c (g233c) virus at MOI = 4. Samples were amplified using primers targeting known regions in the LUNA promoter and UL144 promoter and expressed as a logarithmic function over the Input. H3K4me3 enrichment is represented in **A.** TLR4 was used as a positive control candidate for H3K4me3 enrichment. H3K9me3 enrichment is represented in **B**. ZNF180 was used a positive control candidate for H3K9me3 enrichment. Bars represent averages of triplicate analyses from two independent experiments, with standard deviation shown. Student's *t* test was used to determine the significance of the differences in histone enrichment between infections with Rev and g233c viruses. Points with no error bars represent averages of triplicate measurements from a single experiment.

6.2.1. Activation of latency-associated viral gene promoters is associated with the deSUMOylase activity of LUNA

Previously in Chapter 5, the expression of LUNA protein in Kasumi-3 cells was shown to transactivate latency-associated viral gene promoters derived from HCMV *UL144* and *LUNA*. While a similar effect was also observed using THP-1 cells, where PML protein had been depleted by shRNAs, only the UL144 promoter, but not the LUNA promoter was found to be responsive to LUNA expression. Together, this suggested that LUNA was able to regulate the activity of latency-associated viral gene promoters, likely, by mediating the disruption of ND10 structures; however, in some cases, LUNA may have been able to exert its effect via an alternative mechanism which was independent of ND10 disruption. In order to reinforce the view that LUNA-mediated promoter activation was linked to its ability to disrupt ND10 structures, the same reporter assays were performed in transfected cells as detailed but using the $LUNA_{g233c}$ catalytically dead mutant.

Consistent with the notion that the gene activatory function of LUNA was dependent on its ability to act as a deSUMOylase, co-transfection of the $LUNA_{g233c}$ mutant in Kasumi-3 cells failed to produce any effect on the LUNA promoter (Figure 6.7). However, expression of $LUNA_{g233c}$ was associated with an increase in promoter activity for the UL144 promoter (Figure 6.7). Although this effect did not reach the same level of magnitude as that obtained by co-transfection of wild type LUNA, that the UL144 promoter was responsive to the expression of the $LUNA_{g233c}$ does indicate that LUNA may also partially function in a deSUMOylation-independent manner, insofar as the UL144 promoter is concerned (Figure 6.7). This specific trend was consistent in transfection assays performed with both $LUNA_{g233c}$ and GATA-2, whereby the only additive effect on promoter activity was observed for the UL144 promoter, but not the LUNA promoter (Figure 6.8).

Additional transient co-transfection experiments with the $LUNA_{g233c}$ mutant were also performed using control THP-1 cells (siC) and THP-1 cells with PML knocked down by shRNA technology (siPML). Despite a lack of statistical significance, a trend was observed indicating that co-transfection of $LUNA_{g233c}$ did not yield any change in activity of the LUNA promoter in either siC or siPML cells, unlike co-transfection of wild-type LUNA, which produced a small positive upregulatory effect. This finding suggests that LUNA can activate its own promoter and that this is dependent on its deSUMOylase activity in cells with ND10. By contrast, interrogation of the activity of the UL144 promoter gave a different set of

results, this time with positive upregulation being reported in both PML-positive and PML-negative cells following co-transfection of $LUNA_{g233c,}$ which was comparable to that of wild-type LUNA. This finding suggests that LUNA is also able to upregulate the activity of the UL144 promoter through a mechanism that does not depend on its deSUMOylase activity and hence the need for disrupted ND10. To account for the differences (compared to the LUNA promoter), in magnitude in the response of the UL144 promoter to $LUNA_{g233c}$ in siPML cells compared to siC cells it is possible that the absence of ND10 may promote the ease by which $LUNA_{g233c}$ upregulates the activity of this particular promoter.

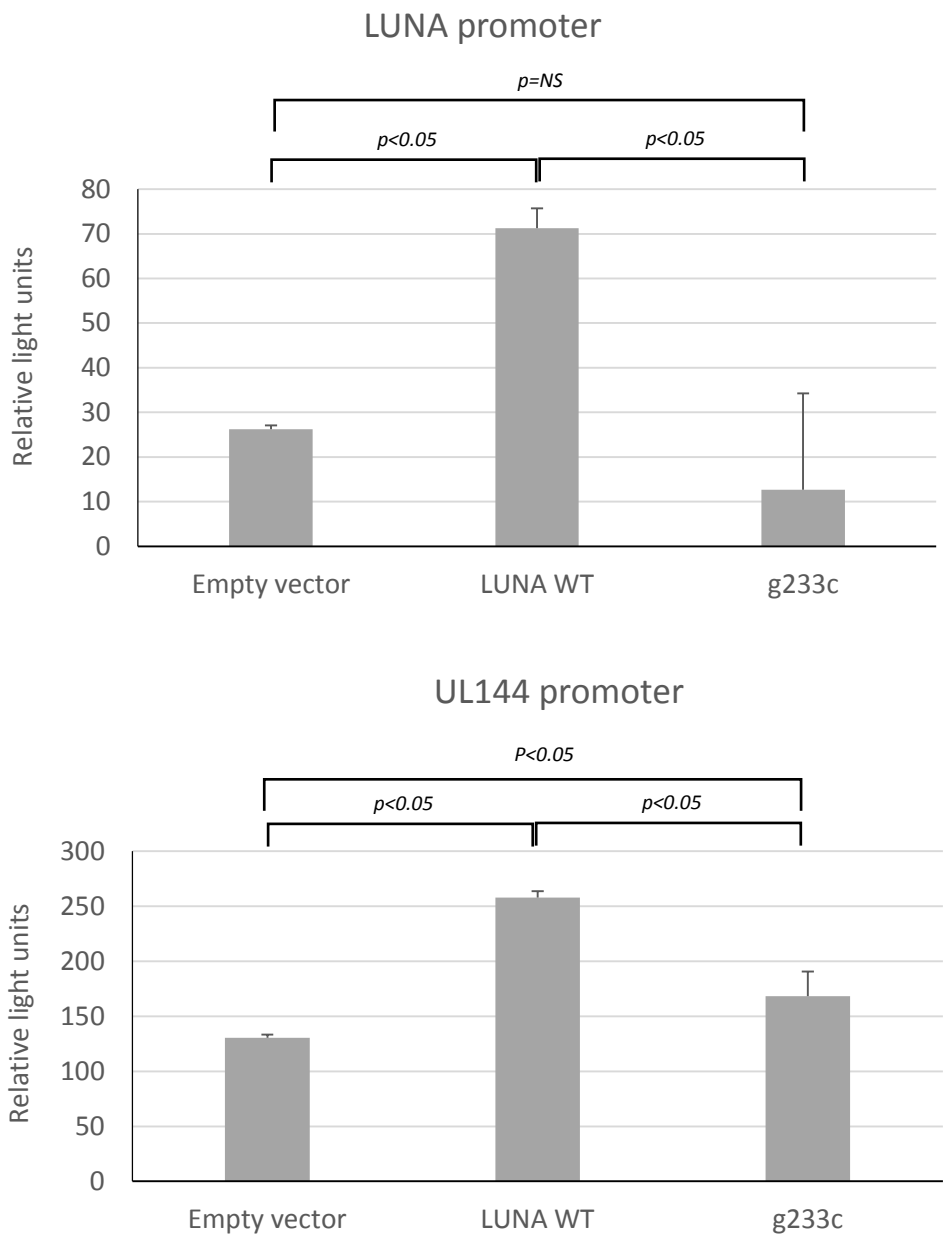


Figure 6.7. The LUNA g233c mutant protein does not exert an effect on the HCMV LUNA promoter, but yields a moderate increase in activity of the UL144 promoter

The $CD34^+$ myeloblastic cell line Kasumi-3 was transiently co-transfected with luciferase reporter plasmids (pGL3) under the control of latency-associated viral gene promoters and effector expression vectors (pCMV-Tag1A) containing triple FLAG-tagged $LUNA_{g233c}$ (g233c). Cells were harvested 48 h post transfection, then assayed for luciferase expression. Results are given as background-subtracted relative light units (RLU). Error bars denote the standard error of samples measured over three independent experiments.

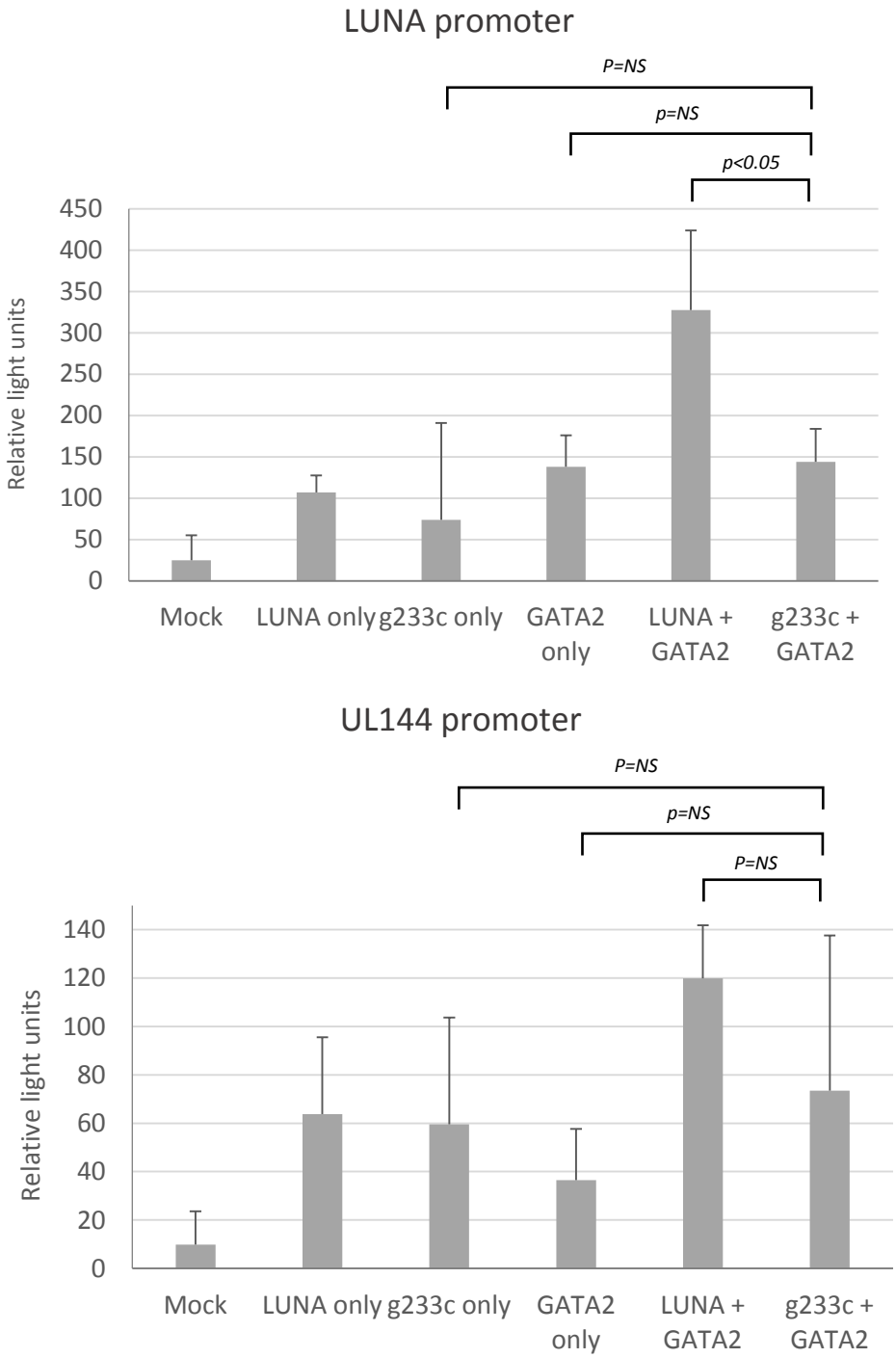


Figure 6.8. Co-transfection of $LUNA_{g233c}$ mutant and GATA-2 yields a combined, additive effect only on the HCMV UL144 promoter, but not the LUNA promoter

Kaasumi-3 cells were co-transfected for 48 h with luciferase reporter plasmids (pGL3) under the control of the LUNA promoter (A) or UL144 promoter (B), along with effector vectors pCMV-Tag1A containing empty (Tag1A) or Triple-Flag-tagged $LUNA_{g233c}$ (g233c); and pEF3.1 containing empty (pEF) or wild type GATA-2 (GATA-2) cDNA. Results are given as relative light units. Samples were background-substracted. Data presented are pooled from 3 biological replicates, with bars representing the averages of duplicate measurements, and standard deviations shown. Student's *t* test was used to determine the significance of differences in promoter activity between g233c + GATA-2 against g233c only and GATA-2 only and LUNA + GATA-2 transfections.

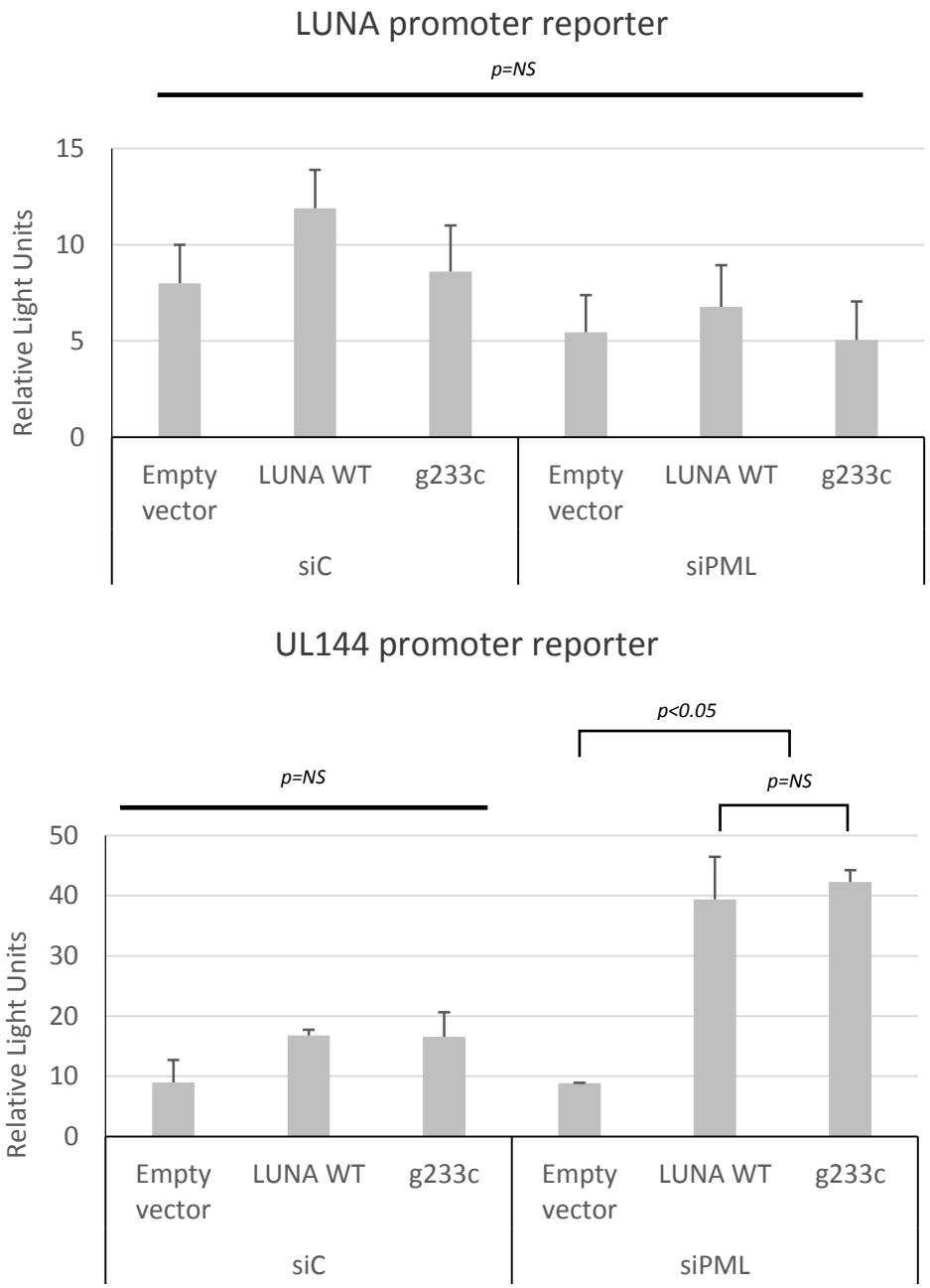


Figure 6.9. In the context of PML protein depletion, only the UL144 promoter, but not the LUNA promoter, is responsive to LUNA g233c mutant expression

THP-1 cells stably expressing either functionally inactive shRNA (siC) or shRNA to PML (siPML) were transient co-transfected with luciferase reporter plasmids (pGL3) under the control of latency-associated viral gene promoters and effector expression vectors (pCMV-Tag1A) containing FLAG-tagged $LUNA_{g233c}$ (g233c). Cells were harvested 48 hours post transfection, then assayed for luciferase expression. Results are given as background-subtracted relative light units. Data presented are pooled from 3 biological replicates, with bars representing the averages of duplicate measurements, and standard deviations shown. Statistical analysis was by one-way ANOVA (LUNA reporter: $F = 1.44$, d.f. = 11, $P > 0.01$; UL144 reporter: $F = 15.70$, d.f. = 11, $P < 0.01$), using a post-hoc Tukey's multiple comparison test. For samples containing LUNA reporter, no statistical significance was observed between any of the groups. For samples containing UL144 reporter, only results generated from transfection of g233c expression vectors into siPML cells were statistically significant compared to all other groups, except LUNA WT.

6.3. DISCUSSION

The results presented in this chapter build upon the previous functional characterisation of LUNA by demonstrating that the protein possesses an isopeptidase activity that allows it to act as a deSUMOylase (Figures 6.1 and 6.2). This activity appears to be critical for the ability of LUNA to disrupt ND10 structures (Figure 6.3). Furthermore, it is also important for regulating viral gene expression during HCMV latency, as transcription and epigenetic analyses of latency-associated viral gene promoters using the g233c mutant virus provided similar results as a LUNA deficient mutant (Figures 6.5 and 6.6). However, at the level of transcriptional activation, the deSUMOylase activity of LUNA appears to be crucial for transactivation of the LUNA promoter, but not the UL144 promoter (Figures 6.7 and 6.8). This indicates that for these promoters in particular, the regulation of the LUNA promoter requires ND10 disruption, whereas for the UL144 promoter, it is possible that LUNA may facilitate regulation via an alternative mechanism that is not deSUMOylase-dependent. Together, these data strengthen the notion that LUNA mediates its regulatory effects on some latency-associated viral gene expression during latent infection by promoting the disruption of ND10 bodies, but may also facilitate similar regulation through another as yet unidentified means. When similar assays were performed using THP-1 cells, expression of the $LUNA_{g233c}$ mutant also gave a similar trend of result as Figures 6.7 and 6.8, with the LUNA promoter showing on upregulation of activity, but activation seen for the UL144 promoter, which was statistically significantly in the absence of PML. This also suggests that the functionality of LUNA may depend on its ability to disrupt ND10, but also by some other deSUMOylase-independent means, whose effect varies according to the target promoter. The greater magnitude of upregulation seen in THP-1 cells by transfection of $LUNA_{g233c}$ on the UL144 promoter, compared to that seen in Kasumi-3 cells, may be accounted for by core differences in properties between the two cell types (i.e. myelomonocytic vs $CD34^+$ phenotypic, respectively). Overall, whilst the above data warrants further investigation into mechanism by which LUNA may mediate latency-associated viral gene expression in the context of ND10 disruption as well as inhibition of deSUMOylation, it will also require further analysis using a greater range of candidate promoters.

7. Investigating human silencing hub (HUSH) complex and its potential role in regulating HCMV latency

7.1. INTRODUCTION

The findings reported throughout this investigation support the role of LUNA in regulating HCMV gene expression during latency, likely, mediated by its novel deSUMOylase activity that allows it target cellular ND10 structures for disruption. Given that cellular ND10 are but one example of factors which contribute towards the intrinsic restriction of herpesviruses, I also reasoned that it was also of particular relevance to consider the impact of other factors that may also be involved in inhibiting HCMV lytic gene expression, specifically, in regards to latent infection.

Recently, in a study performed on the regulation of transgene silencing of retroviruses, Tchasovnikarova *et al*. identified a novel chromatin-associated complex that spreads repressive histone modifications onto newly inserted retroelements[323]. This complex, termed the human silencing hub (HUSH), is composed of three proteins, the chromodomain protein M-phase phosphoprotein 8 (MPP8), the transgenic activation repressor (TASOR) and Periphilin 1 (PPHLN1)[324–326]. Through its baseline interaction with heterochromatin, HUSH directly recruits the chromatin modifier SET domain, bifurcated 1 (SETDB1), to mediate the deposition of H3K9me3 over new genomic insertions[323,327]. Such spreading of heterochromatin from adjacent sequences provides a mechanistic explanation for the related phenomenon observed in *Drosophila* known as positional effect variegation[328]. Recently, KRAB-associated protein 1 (KAP1), a well-known host co-repressor involved in regulating multiple aspects of mammalian homeostasis, was reported to play a role in HCMV latency by directing the recruitment of SETDB1 and HP1 to the viral genome, resulting in the deposition of H3K9me3 marks and transcriptional silencing[244]. However, whether HUSH may also contribute towards SETDB1-mediated silencing of viral genomes remains to be determined. Consequently, the aim of this investigation was to evaluate the role of the HUSH complex in regulating virus gene expression during latent HCMV infection of primary myeloid cells.

7.2. RESULTS

7.2.1. Knockdown of the HUSH complex and SETDB1 can be performed in vitro using shRNA-expressing vectors

Prior to assessing the role of the HUSH complex, it was first necessary to develop a method of eliminating its function *in vitro*. To achieve this, shRNA-mediated knockdown of components of HUSH, and the key HUSH-associated protein, SETDB1, was performed using lentiviral vectors that had been developed by Richard Timms and Daniel Greaves (Lehner group; University of Cambridge). Efficient knockdown of HUSH components, TASOR, Periphilin 1 and MPP8, in addition to SETDB-1 in THP-1 cells was observed following transduction of the above constructs, supporting their use in further experiments (Figure 7.1).

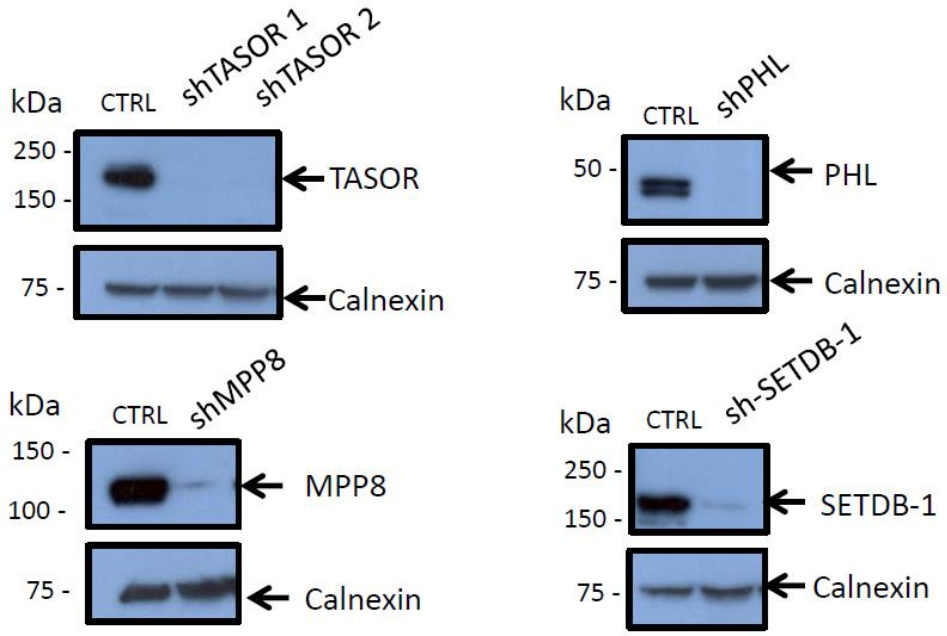


Figure 7.1. Transduction of shRNA targeting components of HUSH and SETDB-1 results in their respective knockdown in THP-1 cells

THP-1 cells were transduced with lentiviruses expressing shRNA against the following components of HUSH, TASOR (shTASOR), Periphilin 1 (PHL) and MPP8 (shMPP8) as well as SETDB-1 (shSETDB-1). Vectors expressing scrambled shRNA was used as a control (CTRL). After 48 hours post-transduction, cells were harvested for western blotting, which was performed in collaboration with Richard Timms and Daniel Greaves (Lehner group).

7.2.1. The HUSH complex is not required for the maintenance of HCMV latency

To determine whether the HUSH complex was essential for HCMV latency, $CD34^+$ cells were first latently infected with an IE2-YFP tagged virus. Next, these cells were targeted by lentiviral vectors expressing shRNA targeting HUSH components and levels of reactivating lytic IE2 gene expression were quantified by immunofluorescent staining. The rationale for this experiment is supported by the fact that after HUSH-mediated repression is established, there is an ongoing requirement for the complex to maintain the state of epigenetic repression[323]. As such, it has been proposed other opposing mechanisms may be present that seek to reverse the heterochromatic state brought about through HUSH, such as a competing active H3K9me3 demethylase activity[329].

Following transduction of shRNA targeting TASOR (shTAS) and SETDB1 (shSET), compared to shRNA controls (shControl), the numbers of IE2-YFP expressing foci showed a marginal increase (Figure 7.2). This initially suggested that components of the HUSH complex could play a minor role on the suppression of lytic gene expression during latency. However, it should be bone in mind that in this analysis, confirmation of the levels of expression of SET and TASOR were not analysed due to a lack of available $CD34^+$ cell samples and equivalent efficiency of TASOR and SET knockdown was assumed on the basis of data presented in Figure 7.1.

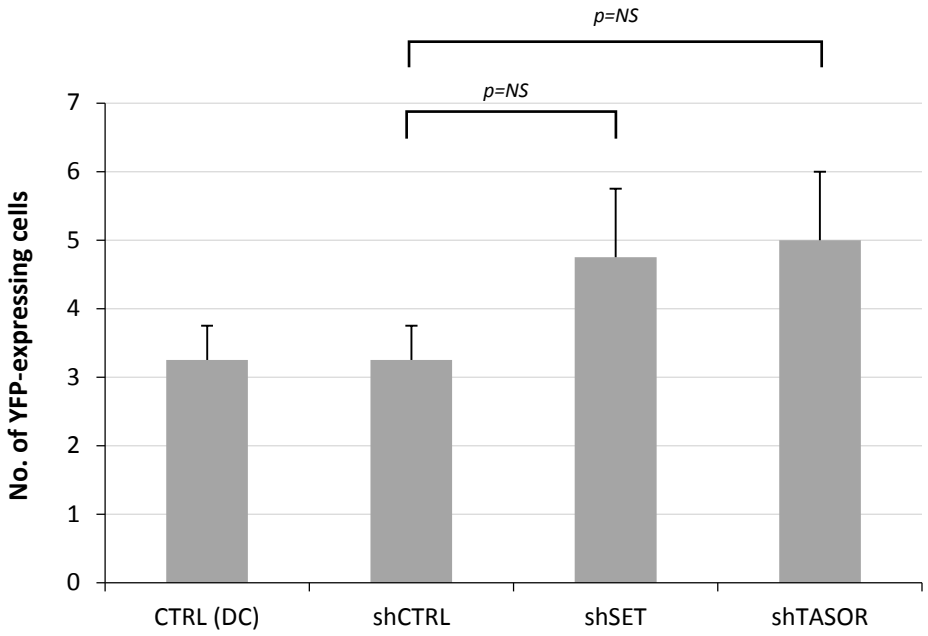


Figure 7.2. Transduction of shRNA targeting HUSH components has little effect on reactivation of latently infected CD34$^+$ cells

Primary CD34$^+$ cells were latently infected with TB40-BAC4 IE2-YFP tagged virus at MOI = 5 and incubated for 3 days to establish latency. Cells were then transduced with lentivirus expressing shRNA targeting TASOR (shTAS), SETDB1 (shSET) or scrambled shRNA (shCTRL), and monitored for YFP-expressing foci. The relative number of YFP-expressing cells were enumerated using ImageJ software. Values represent averages of 5 fields of view of 100 cells, each with SD error bars. Counts were made 15 days post-infection. Student's *t* test was used to determine the significance of differences in YFP-expressing foci between shCTRL and shSET or shTASOR conditions.

7.2.2. Components of the HUSH complex are not associated with epigenetic regulation during latency

To interrogate the effect of HUSH-mediated regulation on HCMV latency further, I performed ChiP assays on the same latently infected $CD34^+$ cells which were subjected to HUSH knockdown in order to analyse levels of histone modification surrounding the MIEP.

No discernible trend was observed consistent with these factors affecting chromatin marks around the MIEP in latently infected cells, suggesting that HUSH is not critical in regulating HCMV latency (Figure 7.3). Notably, levels of H3K4me3 enrichment, a marker of transcriptional activation, showed no significant difference between the samples relative to control shRNA. Although repressive H3K9me3 levels were lower in cells transduced with shSET than shTASOR, relative to control, no clear result may be drawn from this owing to a lack of statistical power.

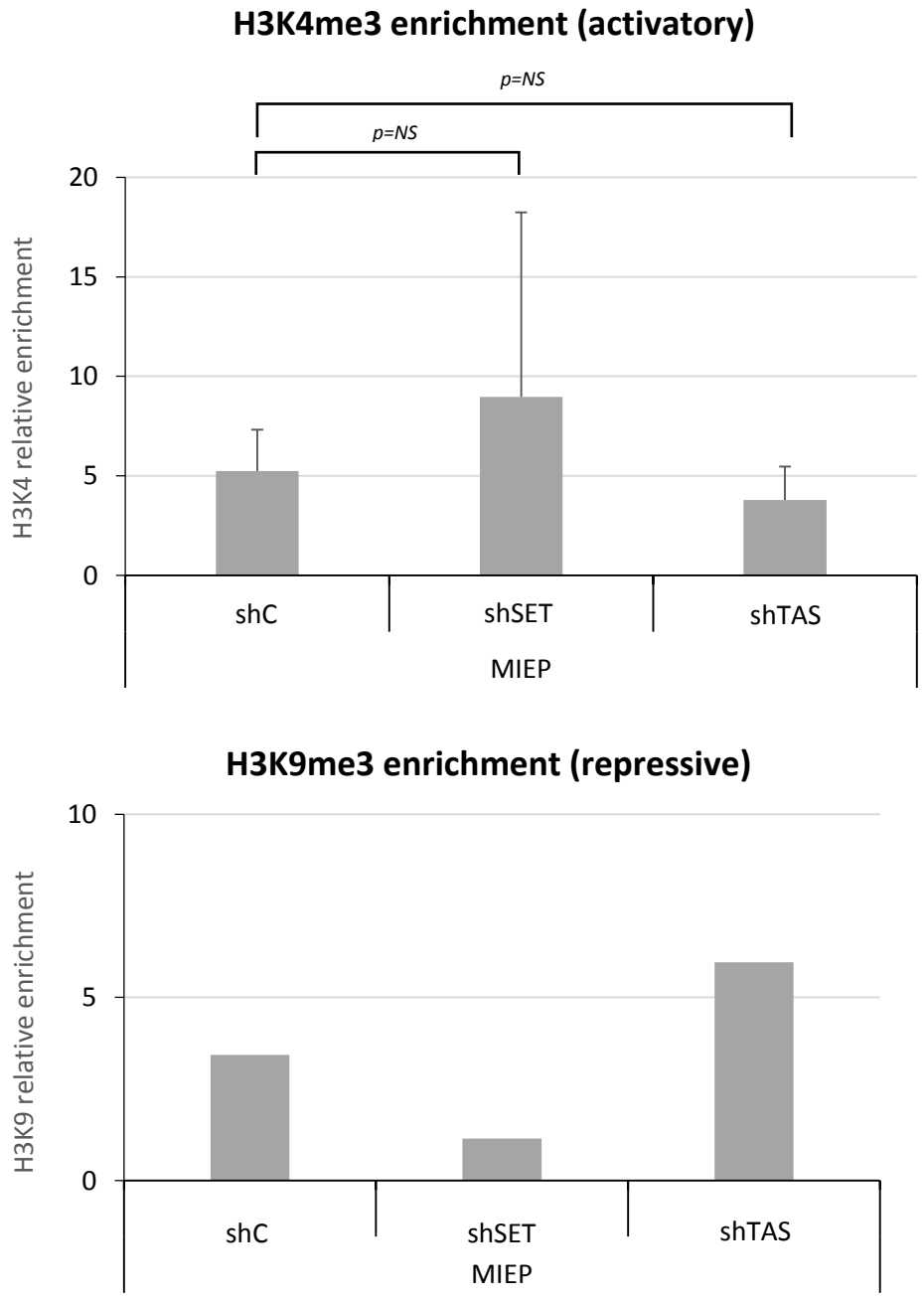


Figure 7.3. Transduction of shRNA targeting HUSH components and SETDB1 yields no significant changes in the chromatin landscape over the MIEP of latently infected cells

$CD34^+$ cells previously infected with TB40-BAC4 IE2-YFP tagged virus were harvested after 10 days post-transduction of shRNA for ChIP analysis. Assays were performed using anti-H3K4me3, anti-H3K9me3 or isotype control (IgG) antibodies. Samples were amplified using primers targeting known regions in the MIEP and expressed as a logarithmic function over the Input. H3K4me3 enrichment is shown in **A.** H3K9me3 enrichment is shown in **B**. Bars represent averages of triplicate analyses from two independent experiments, with standard deviation shown. Student's *t* test was used to determine the significance of the differences in histone enrichment between transductions with shC against shSET and shTAS. Points with no error bars represent averages of triplicate measurements from a single experiment.

7.2.3. Knockdown of HUSH yields no effect on the activity of the MIEP in early myeloid cells

Presently, it is understood that only newly inserted retroelements (transgenes) may be subjected to HUSH-mediated silencing. To that end, it may be the case that HUSH is not involved in the regulation of HCMV latency, given that such viral episomes do not undergo integration into the host genome. Indeed, this scenario is consistent with the data presented so far in Chapter 7. Nonetheless, to explore this further in the context of HUSH complex function, an experiment was performed to compare levels of activity of MIEP-reporter in THP-1 cells between those which had either had the reporter integrated into the host genome or introduced by transient transfection.

Following transduction of shRNA targeting HUSH or SETDB1, THP-1 cells containing the integrated reporter showed a significantly raised level of MIEP activity, relative to shRNA controls (Figure7.4A). However, in contrast to cells where the MIEP-reporter had been inserted as non-integrated plasmid, no significant increase in activity was observed compared to shRNA controls, as indeed, they were found to show a counterintuitive repression (Figure 7.4B). Together, these results indicate that non-integrated viral episomes are not likely to be affected by HUSH-mediated repression.

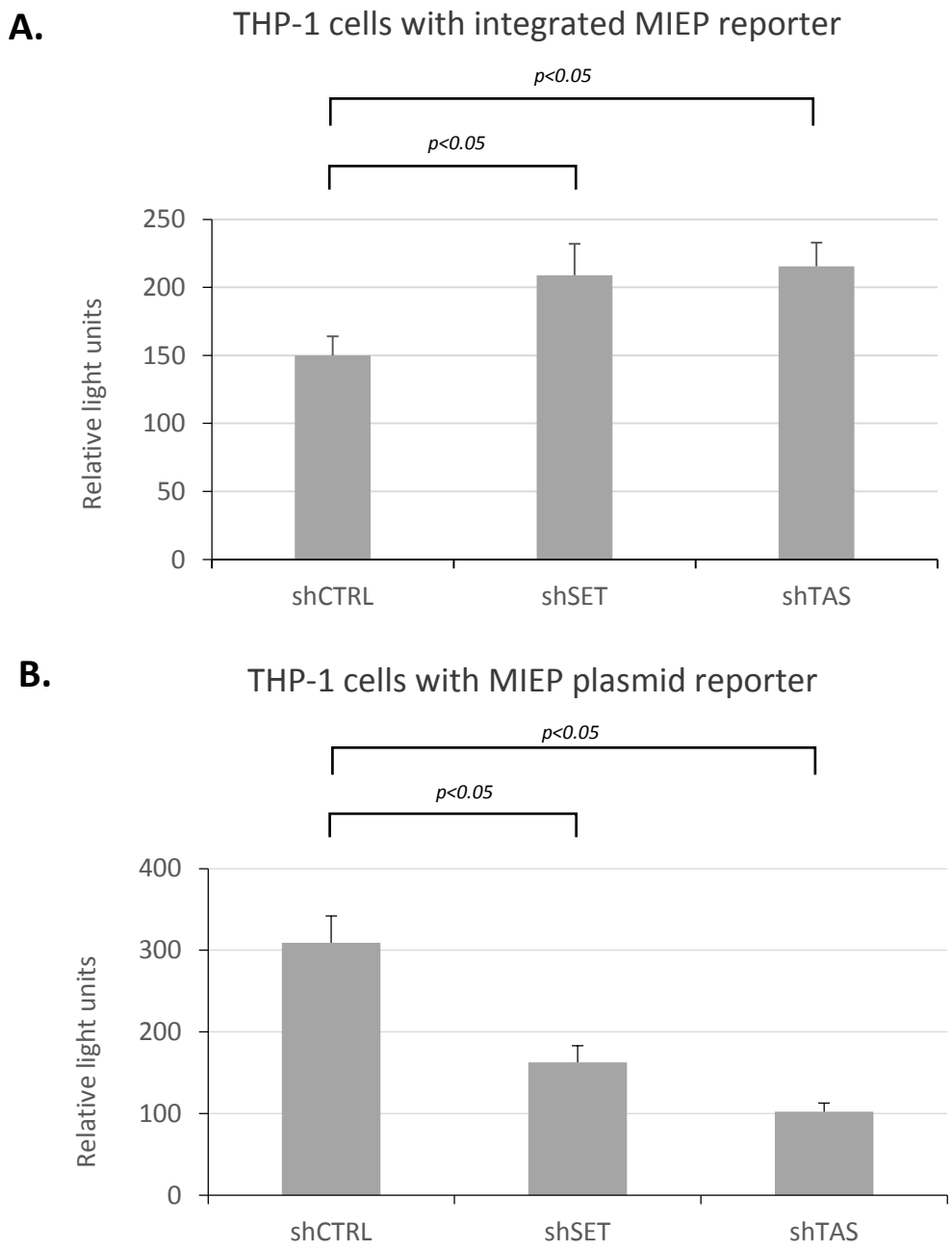


Figure 7.4. Knockdown of HUSH in THP-1 cells containing stably integrated reporters show increased levels of MIEP activity, as measured by luciferase expression, but this effect is not observed in cells containing plasmid reporters that have been introduced by transient transfection

Luciferase assays were performed to measure the effect of HUSH knockdown on the activity of the HCMV MIEP in THP-1 cells. MIEP luciferase-based reporters were introduced into THP-1 cells either by genetic modification in order to generate cells containing stably integrated constructs (A) or by transient transfection using separate plasmid DNA (B). Knockdown of HUSH was performed using lentivirus transduction; shRNA targeting TASOR (shTAS), SETDB1 (shSET) or scrambled shRNA (shCTRL). Data presented are pooled from 3 biological replicates, with bars representing the averages of triplicate measurements, and standard deviations shown. Student's t test was used to determine the significance of differences between shCTRL and shSET or shTAS.

7.3. DISCUSSION

Together, the findings of this investigation demonstrate that the HUSH complex is not likely to be involved in regulating HCMV latency with respect to suppressing lytic IE gene expression. Although immunoflorescence data demonstrates a weak trend implicating HUSH in the maintenance of latent infection, results from epigenetics analysis suggest that HUSH does not play a significant role overall. Indeed, this was further supported by the observation that viral MIEP-reporters which have been integrated into the host genome appear to be subjected to HUSH-mediated regulation (in that knock-down of HUSH components activated the MIEP), whereas transiently transfected MIEP reporters introduced as separate, circular plasmids – perhaps akin to viral episomes formed during infection – are not. Why knock-down of HUSH components leads to an apparent decrease in activity of transiently transfected MIEP-reporters remains unclear from the point of view of these analyses. One possible explanation is that the knock-down of HUSH components leads to an indirect effect that increases the expression of other cellular transcriptional repressors that can target vector-based MIEP-reporters and inhibit their overall level of luciferase expression. Overall, although HUSH serves as a potent host transcriptional repressor, the manner by which it functions does not appear to apply to HCMV with respect to normal conditions of infection. To this end, other known cellular regulators, such as early myeloid-associated transcription factors, may be directly responsible for mediating the formation of a repressed chromatin structure over the MIEP during latent infection.

8. Conclusions

The principle aims of this thesis were to evaluate the functional significance of a single HCMV latency-associated gene, LUNA, with respect to its regulatory effects on the latently infected cell. Much of the work is detailed across four investigative chapters, with a fifth chapter focussing on more exploratory research. The first chapter centres on the impact of latent HCMV infection on the host cell, in particular, examining whether cellular ND10 structures – known intrinsic inhibitors of herpesvirus replication – are disrupted as a result of latent infection and whether the viral latency-associated protein, LUNA, may be responsible for producing such a phenotype. The second chapter interrogates the putative regulatory function of LUNA (in lieu of its ability to promote the disruption of ND10) and assesses whether the expression of LUNA is associated with changes in virus gene transcription during latent infection. The third chapter examines the effect of LUNA on the activity of latency-associated viral gene promoters in order to suggest a possible mechanism by which LUNA may mediate transcriptional regulation. The fourth, penultimate chapter seeks to address whether the functionality of LUNA as a viral transcriptional regulator is provided by a novel isopeptidase activity (encoded within its C-terminal domain), which enables the protein to target ND10 for disruption. Finally, the fifth chapter enquires after the role of another set of host repressors in regulating HCMV latency through a distinct process known as transgene repression.

In the first investigative chapter (Chapter 3), I showed that latent HCMV infection resulted in the disruption of ND10 structures, as revealed by a relative absence of PML-containing foci in latently infected early myeloid cells compared to uninfected controls. This novel finding indicates that viral-mediated disruption of ND10 occurs in both phases of the virus life cycle, where such disruption has only previously been observed in the context of lytic infection. Crucially, it now appears that the HCMV LUNA protein, a viral latency-associated factor may also be involved in disrupting ND10, specifically, when it is expressed under conditions that support latency. The mechanisms underlying this may differ from those described for the lytic genes, IE1 and pp71, which are also known to disrupt the functions of ND10. Broadly speaking, the disruption of ND10 during lytic infection fits with the notion of the virus overcoming the negative repressive effects instigated by a host intrinsic defence mechanism, such that productive infection of permissive cell types is not inhibited. By contrast, the apparent loss of these structures during latency is surprising, given that their presence is otherwise thought to contribute to the repression of viral genomes, an important criterion for

silencing IE gene expression and maintaining the virus in a latent state. Consequently, other regulatory mechanisms, beside the manifestation of ND10, are more likely to be implicated in controlling viral latency. Nevertheless, by allowing the virus to more efficiently express its genes, the disruption of ND10 during latent infection may serve a similar purpose as that for lytic infection. Indeed, it is well established that viral latency is dependent on the ability of HCMV to successfully undergo virus gene expression and any event that may help the virus to facilitate this should not be discounted.

The next investigative chapter (Chapter 4) sought to determine whether LUNA played a putative role in regulating virus gene expression during latency. Using models of experimental latent infection based on primary myeloid cells, mutant Merlin viruses deficient in LUNA were analysed for their ability to express both latency-associated and lytic viral genes. In cells infected with LUNA knockout viruses, a clear lack of gene expression for the latent viral gene product UL138, and lytic IE1, was observed compared to those infected with wild type viruses. This finding indicates that LUNA is required for enabling efficient virus gene expression to occur during latency. Importantly, because UL138 is understood to be required for the maintenance of latent HCMV infection, the ability of LUNA to normalise UL138 expression during latency also provides evidence of its role in contributing towards this process. To account for the increased levels of MIE gene expression, it is conceivable that LUNA, by disrupting ND10, may be relieving the MIEP of a number of transcriptional repressors involved in targeting it for silencing. While ND10 disruption does not appear to be sufficient for viral reactivation from latency, likely because permissiveness for lytic infection remains intimately tied to the differentiation status of the host cell, there is ongoing evidence to suggest that LUNA is involved in promoting efficient viral reactivation[317]. In addition to the above findings, it was also shown that LUNA expression during latent infection coincided with the acquisition and loss of markers of transcriptional activation and repression, respectively, over the promoters of latency-associated viral genes. This observation highlights the impact of LUNA on molecular mechanisms governing latency-associated viral gene expression, which correlate with changes observed downstream at the mRNA level. Taken together, these results help to confirm that LUNA plays a role in regulating latency-associated virus gene expression during latency.

To further characterise the function of LUNA during latency and build upon the investigations outlined in Chapter 4, the following series of experiments (Chapter 5) aimed to assess whether direct expression of the protein *in vitro* was associated with alterations in levels of viral

promoter activity. Given that such changes should correspond with the ability of LUNA to augment viral gene expression, any resulting findings might explain how LUNA-mediated increases in virus gene expression could be mediated by the modulation of viral transcriptional output. Using a reporter assay system established in early myeloid cell lines, levels of activity from the promoters of two latency-associated viral genes, *UL144* and *LUNA*, were measured in the context of transient expression of wild-type LUNA. In the presence of LUNA, both viral gene promoters showed a significant increase in activity, indicating that LUNA was able to exert a positive upregulatory effect on latent viral gene transcription, including positive autoregulation of its own promoter. This suggests that LUNA is able to augment latency-associated viral gene promoters in order to affect alterations in virus gene expression. Importantly, the finding that the LUNA promoter does not show a significant difference in activity following LUNA expression in PML-knockdown cells argues for the ability of LUNA to exert its effects through the disruption of ND10. Even in the absence of ND10, however, it is noteworthy that the UL144 promoter was responsive to LUNA expression, indicating that LUNA may also promote viral gene transcription via an alternative mechanism that does not necessarily involve ND10 targeting. Earlier investigations concerning the regulation of latency-associated viral gene expression revealed that the cellular transcription factor, GATA-2, is able to regulate the activity of both the LUNA and UL144 promoters to suggest a potential mechanism by which latent viral gene expression may be induced by the host, prior to any *de novo* viral protein synthesis. When both GATA-2 and LUNA were co-expressed in the above system, this was found to yield an additive effect on the LUNA promoter, as well as the UL144 promoter suggesting that these factors may cooperate to enhance LUNA and UL144 gene expression during latent infection. These factors also interact physically with one another, which may be important for enabling their combined effect upon viral promoter activity.

In the penultimate investigatory chapter (Chapter 6), the capacity of LUNA to function as a viral transcriptional regulator was tested against its ability to act as a viral isopeptidase. In brief, the LUNA protein had been found to possess a putative active site that shared weak homology to a class of cellular enzymes involved in the ubiquitin-like modification of proteins (Poole *et al*. Under review). Here, it was shown that LUNA could participate in the related post-translational modification of SUMO, specifically, by deconjugating SUMO moieties from protein substrates (deSUMOylation). When the active site was mutated to create a catalytically defective LUNA mutant ($LUNA_{g233c}$), subsequent transient transfection analysis in early myeloid cells revealed that the mutant failed to disrupt ND10,

indicating that the protease activity in question was required for LUNA to target ND10 for disruption. Notably, the expression of g233c was not associated with an increase in activity of latency-associated viral gene promoters relative to wild-type LUNA expression, suggesting that the deSUMOylase function was also required for normal HCMV promoter activity. Consistent with this, primary myeloid cells infected with g233c-containing mutants (g233c) failed to efficiently express viral genes compared to wild-type phenotype controls, akin to that observed for LUNA knockout viruses. Combined, these findings are in keeping with the main hypothesis that the disruption of ND10 by LUNA is important for the regulation of viral gene expression. However, as alluded to earlier, some latency-associated viral genes appear to be regulated by other mechanisms irrespective of LUNA-mediated ND10 disruption. This is reflected by the fact that g233c expression in cells depleted of PML appears to result in an upregulation of viral UL144 promoter activity, but does not yield any effect on the LUNA promoter; however, additional analysis is required to establish whether these particular findings are statistically significant.

In the final results chapter (Chapter 7), an investigation was performed to evaluate the impact of another potential regulator of HCMV latency, specifically, concerning a host-derived factor termed HUSH. Briefly, HUSH is a recently characterised chromatin-associated complex that is involved in transgene repression, where it mediates the spread of pre-existing heterochromatin[323]. Using latently infected $CD34^+$ myeloid cells, the contribution of HUSH with respect to latency was determined by shRNA-mediated depletion. Following transduction of shRNA targeting HUSH component TASOR and the key effector SETDB, a small increase in IE gene-expressing foci was observed, suggesting a potential role for HUSH in regulating latent HCMV infection. However, subsequent analysis of the chromatin landscape revealed little, if no significant change in the levels of activatory or repressive histone modifications to indicate that HUSH is not likely to be important for the regulation of the viral MIEP during latency. The significance of this finding is made clear by the fact that levels of MIEP activity show no difference when HUSH components are depleted, unless the MIEP has been experimentally integrated into the host cell genome, a context that is not present under normal conditions of infection. While these do not appear to accommodate a role for HUSH in the maintenance of latency, it is possible that they may still play a role in regulating MIEP activity during latent infection and this should be investigated further.

The general thesis presented in this dissertation is that HCMV promotes the disruption of ND10 structures, an intrinsic cellular defence mechanism, during latent infection, which as a

result, appears to enhance the activity of its own latency-associated transcriptional programme. The findings presented contribute further to the discussion that HCMV latency is a dynamic process, one composed of a series of regulatory interactions between the virus and host cell that lie apart from the necessary repression of lytic genes. When placed into a wider context, ND10 structures should continue to be regarded as fulfilling a potent antiviral role that specifically calls for their abrogation by HCMV[330]. As such, numerous studies have firmly established the capacity of ND10 to thwart robust viral replication through individual contributions made by their major constituent proteins, PML, hDaxx and Sp100, which function as host restriction factors[331]. Indeed, PML and Sp100 were recently shown to act as IFN-stimulated genes (ISGs) that become upregulated during HCMV infection, further indicating that enhancement of the presence of ND10 and its associated effects is likely to fall under the rubric of the host IFN antiviral response[332,333]. Consequently, it is unsurprising that during the early stages of lytic infection, a number of strategies are employed by the virus to counteract the restriction activity of ND10, with the viral proteins IE1 and pp71 serving key roles in this regard[181]. Yet, despite the ability of ND10 to suppress virus gene expression, it is noteworthy that a number of studies have indicated a lack of involvement of ND10 in the establishment of HCMV latency. Notably, in cellular settings of latent infection, hDaxx had previously been shown to contribute towards critical repression of the MIEP through the recruitment of HDACs[197]. However, in contrast, knockdown of hDaxx in undifferentiated NT2 cells was found not to be sufficient to trigger IE gene transcription[185]. Additionally, it was recently reported that depletion of each of the above ND10 components in undifferentiated THP-1 cells had no effect on IE gene expression; but, compared to control cells, did dramatically increase the efficacy of viral reactivation following differentiation[302]. Together, the above findings support the likelihood of ND10 playing a more prominent role in restricting lytic replication and reactivation, rather than supporting latency; though, they failed to bring into question the possibility of ND10 being targeted for disruption during the latent period itself and by extension their potential complicity in restricting latent carriage. Here, my study into the function of the latency-associated gene product, LUNA, sheds light on this specific issue, characterising the protein as a viral factor responsible for mediating such disruption during latent infection, similar to what has been observed in cases of lytic replication. One may therefore infer that HCMV has evolved to overcome ND10 as a blanket requirement for infection, since the virus possesses the means to disrupt these structures in the host cell irrespective of its level of permissiveness for viral replication. During latency specifically, the disruption of ND10 has been shown to be important for enabling latency-associated viral gene expression, as evidenced by reduced levels of UL138 and IE

mRNA detection from infected early myeloid cells when LUNA is either absent or mutated. In conjunction with this, decreases in the activities of latency-associated viral gene promoters, associated with corresponding changes in the surrounding epigenetic landscape, are also observed when LUNA is absent or mutated. Such findings are consistent with previous investigations into LUNA, which demonstrated its requirement for efficient expression of UL138 from infected $CD14^+$ and $CD34^+$ cells, and additionally, show that even latency-associated viral gene promoters are capable of undergoing dynamic changes in chromatin structure during the latent period[266,334]. Thus, the removal of ND10 carries implications in terms of supporting viral latency, but also promoting efficient viral reactivation, as has been recently demonstrated by *Poole et al.* (2018)[317]. Nonetheless, the fact that incoming herpesvirus genomes accumulate at the periphery or within the central core of ND10 at early times post-infection should not be discounted[179,180,335]. Of particular note is the observation that ND10 found associated with herpesvirus genomes do not represent pre-existing sites, but are likely newly assembled structures that become localised to viral DNA, which is in keeping with their ability to be co-regulated by host IFN immune responses[336]. Consequently, since LUNA must be expressed *de novo* to ensure disruption of ND10, and tegument pp71 protein is not present in the nucleus during latent infection, this leaves open the possibility that these structures are still able to impact on HCMV genomes, and hence contribute towards latency. In support of this, ND10 possess the capability to subject HCMV genomes to epigenetic mechanisms of transcriptional control, which may be linked to the formation of inactive chromatin over the MIEP during latent infection, also seen at the start of lytic infection[169,185]. However, further studies will be required to define the exact contribution of individual ND10 proteins to this process and help establish a time frame of regulatory events following viral genome deposition and chromatinisation up to parental ND10 disruption. Here, it may be important to uncover the identities of the various chromatin remodelers that bind to the viral genome when ND10 components are either present or absent, as this may help to further evaluate their roles as key transcriptional regulatory effectors.

Because the loss of ND10 suggests an absence of intrinsic transcriptional repressive activity, this naturally raises the question of what other mechanisms may contribute to the regulation of latency, but, more still, brings into mind the consequences of granting further licence for latency-associated gene expression within the latently infected cell. These considerations are in keeping with the viewpoint that HCMV latency should not be regarded as a period of viral quiescence, but rather one composed of an active, ongoing interplay between the host cell and virus[337]. To begin with, it is generally understood that the nuclear factor milieu within a given

cell type and stage of differential serves as the critical determinant of whether the viral MIEP exhibits an underlying repressive phenotype capable of supporting latency, which contrasts with the milieu of a terminally differentiated cell type that would shift the MIEP towards a constitutive de-repressed phenotype. This is consistent with the fact that the MIEP is known to possess binding sites for a range of host transcriptional repressors as well as activators[102,170]. In conjunction with the data described thus far, it is therefore plausible that the loss of ND10 would have no effect on the acquisition of repressed chromatin found around the MIEP, since the silencing would be dictated by other host mechanisms that take priority in the latently infected cell. Rather, it seems likely that ND10 acts as a second layer of repression that primarily functions in the context of lytic permissiveness, in which the MIEP would conform to an open, transcriptionally active state[170]. Moreover, viral latency-associated gene products are becoming increasingly recognised as contributing importantly to latency as well as other processes necessary to sustain effective latent carriage[338]. Notably, viral UL138 has already been implicated in silencing IE gene expression during latency[339]. Additionally, viral US28 was recently shown to be expressed in latently infected monocytes, playing a role in attenuating cellular signalling pathways that was required for latency establishment[340]. Besides viral protein coding genes, however, viral non-coding RNAs (ncRNAs) are expressed during latent infection and may also act as important regulators of this process, particularly in regards to avoiding immune surveillance. For instance, a possible role has been suggested for miR-UL112-3p in downregulating IE1 gene expression, which could help prevent T cell recognition of latently infected cells[341,342]. Similarly, miR-UL148D-1 targets ACVR1B, part of the activin signalling axis, to limit cellular IL-6 secretion by infected monocytes[315]. Together, the expression of all these transcripts - along with their subsequent positive impact on latent infection – may be augmented by the ability of LUNA to upregulate latency-associated gene promoter activity. Here, LUNA could function either by abrogating the inhibitory effects of ND10, or possibly, by directly affecting the promoters responsible through an as yet unidentified mechanism. Nevertheless, given that the activities of only two candidate latency-associated viral gene promoters were assessed in this project, it is clear that further work on other latent gene promoters will need to be performed to determine the extent of the impact of LUNA. Yet, insofar as the latent targeting of ND10 by LUNA is concerned, this points to a legitimate causal explanation, so as to facilitate viral transcription conducive to latency. One final consideration is how LUNA becomes expressed in the first instance. As reported here and elsewhere is the finding that the HCMV LUNA promoter is responsive to the effects of GATA-2**,** which indicates that the virus is able to exploit host cellular

transcription factors to induce expression of its own genes and thereby regulate its own latency-associated transcriptome post-infection[215].

Turning more closely to the viral LUNA protein itself, the identification of its novel encoded deSUMOylase activity lends fresh perspective to its general functionality, and broadly, the manipulation of SUMO pathways by the virus. Now, for the first time, a putative mechanism of action may be ascribed to the protein by which it may target ND10 structures for disruption, thereby relieving the effects of host cell transcriptional repression in manner that is consistent with previously published attempts to characterise its function. Moreover, although a number of HCMV proteins have since been implicated in the exploitation of SUMO modification system, LUNA also serves as the first example of one that functions within the context of latency. Before this discovery, KSHV was the only human herpesvirus whose ability to modify host SUMOylation during latency was extensively researched. Here, the key master regulator of KSHV latency, LANA1, had previously been shown to interact with SUMO-2 in order to recruit the chromatin remodeler SUMO-2-modified KAP1 along with other proteins to silence lytic genes and maintain the viral episome[343]. In addition, another KSHV latency-associated protein, vIRF-3 (a viral analogue of host interferon regulatory factor 3) was found to block SUMO modification of Rb, p53 and p130 factors, involved in controlling host cell cycle progression, thereby indicating that the virus was able to affect specific SUMO-modified signals[344,345]. Specifically, HCMV LUNA has been shown to possess an isopeptidase activity that allows it cleave SUMO-2/3 moieties, although its overall enzymatic specificity remains unclear[317]. Thus, it will be interesting to further evaluate the effects of LUNA and its associated deSUMOylase activity on viral proteins, but also host ones too, not least as the study of SUMO-regulated pathways is becoming a rapidly developing topic within the herpesvirus field[173,346]. For instance, it has recently been reported that GATA-2 signalling is stabilised by deSUMOylation, which may account for its capacity to function cooperatively with LUNA in regards to regulating latency-associated gene transcription[347]. Moreover, structural analysis of the LUNA protein should be performed to help understand how the protein localises to putative targets and also clarify the precise structure of its active site. This carries obvious implications for the pharmaceutical development of novel compounds to specifically target and inhibit LUNA, as part of a wider therapeutic strategy.

Overall, for the purposes of this dissertation, further work will need to be performed to determine the extent to which the transcription of latency-associated viral genes are affected

by the activities of LUNA. Notably, the repertoire of candidate latency-associated viral gene promoters for interrogation requires expansion. In addition, further evidence of PML disruption should be gathered in order to more directly attribute changes in gene expression to loss of ND10 and, in turn, LUNA expression. This could be achieved by immunoblotting for SUMO-modified PML to detect loss of high molecular weight PML species. Most importantly, it will be worthwhile employing the use of some alternative means of inhibiting the deSUMOylase activity of LUNA, for instance, by making use of currently available pharmacological agents (e.g. pan-isopeptidase inhibitors), to strengthen not only the key hypothesis that part of the ability of LUNA to regulate latent gene transcription is dependent on its enzymatic activity, but also determine whether disabling the function of LUNA could prevent the virus from establishing and maintaining a successful latent infection, thereby, restricting its capacity to reactivate and cause disease.

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